



Characteristics of Enterobacteriaceae in surface water from the North West Province

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University

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DECLARATION

I declare that this dissertation for the degree of Magister Scientiae of Microbiology at North-West University, Potchefstroom Campus, has not been submitted by me for a degree at this or another University, that it is my own work in design and execution, and that all materials contained herein has been duly acknowledged.

Lee-Hendra Elvida Chenhaka

A handwritten signature in black ink, appearing to read 'Lee-Hendra Elvida Chenhaka', written in a cursive style.

Date:

21 March 2025

DEDICATION

I dedicate this work to my beloved mother, **Hermien Stal-Julies**, whose tenacity and perseverance inspire me. You have taught me that with persistence and determination, anything is achievable. Reflecting on my journey, I now see how your example of meticulousness and dedication to cleanliness has shaped my mindset, a principle that resonates deeply in the world of microbiology. Your love, guidance, and steadfast support have been the foundation of my strength and success. I am forever grateful for all you have instilled in me.

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RESEARCH PUBLICATION

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CONFERENCE PRESENTATIONS

- | | | |
|------|--|---------------------|
| 2018 | SASM (South African Society for Microbiology) | Poster Presentation |
| 2019 | ARAE (Antimicrobial Resistance in Animals and the Environment) | Poster Presentation |
| 2023 | EDAR (Environmental Dimension of Antibiotic Resistance) | Poster Presentation |
| 2024 | UESM (Unit for Environmental Sciences and Management) | Oral Presentation |

COMMUNITY ENGAGEMENT PARTICIPATION

2018 – 2023: National Water Week Participation; Envira Report: Valuing water: quality and quantity. *Envira Science Magazine* (2020).

2018 – 2018: Nelson Mandela Day (67 minutes): Environmental Cleanup at Primary School

2018 – 2022: International Microorganism Day; Envira Report

2018 – 2021: World Antibiotics Awareness Week; The International Water Association newsletter: Health Related Water Microbiology Specialist Group (Volume 18, p8-10; November 2018)

2019 – 2019: Water Lekgotla Participation

INTERNATIONAL RESEARCH VISITS

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ABSTRACT

Faecal pollution of surface water systems poses a significant risk to the environment and public health. According to the latest Green Drop report in South Africa, the North West province has been identified as a high-risk area. Contributing factors include rapid urbanisation, inadequate sanitation infrastructure, informal settlements, ageing wastewater treatment plants with minimal maintenance, and the overloading of treatment capacities. Faecal pollution is evidenced by the presence of indicator microorganisms such as coliforms, which highlight the prevalence of *Escherichia coli* (including its pathogenic serovar *E. coli* O157), alongside other Enterobacteriaceae genera, including *Hafnia*, *Klebsiella*, *Enterobacter*, *Citrobacter*, and *Serratia* species. Many of these non-*E. coli* Enterobacteriaceae have gained clinical significance due to their ability to harbour antibiotic-resistant genes (ARGs) and produce extended-spectrum beta-lactamases (ESBLs). These pathogens have the potential to cause diseases such as urinary tract infections, diarrhoea, neonatal meningitis, sepsis, and respiratory infections, posing a severe threat to public health and placing a burden on healthcare systems. Therefore, this study aimed to investigate the characteristics of Enterobacteriaceae isolated from selected surface water.

The study investigated the water quality and microbiological hazards within three key surface water systems in the North West province: the Mooi River, the Schoonspruit River, and the Setumo Dam. Physico-chemical and microbial parameters were measured and compared against the water quality targets recommended by the Department of Water and Sanitation (1996). Alarming findings included highly alkaline pH levels exceeding 9 in recreational sites, posing eye, skin, and mucosal irritation risks, and exceptionally high total dissolved solids (2 197 mg/L) at Site S1 of the Setumo Dam, likely linked to untreated wastewater discharge. Furthermore, total coliform and *E. coli* counts consistently exceeded acceptable thresholds for wastewater effluent and recreational use, with the lowest *E. coli* levels recorded at the Schoonspruit River's dolomitic eye (58 MPN/100 ml), indicative of widespread faecal contamination.

A total of 3 532 *E. coli* isolates were recovered from the water systems, including 818 putative *E. coli* O157 colonies. However, Pearson's correlation analysis ($r = 0.61$, $p = 0.27$) revealed no significant relationship between the overall *E. coli* counts and the presence of *E. coli* O157. Thirty-four haemolytic Enterobacteriaceae isolates, selected for their ability to lyse erythrocytes, were further characterised. Twenty-eight isolates were confirmed to be *E. coli* on EMB agar and exhibited distinct extracellular enzymatic activities, including haemolysin, β -galactosidase, and DNase production. Notably, isolates from the Setumo Dam also exhibited catalase, lipase, and serine protease activity, suggesting adaptations to nutrient availability in this environment. Biochemical profiling identified citrate utilisation and urea hydrolysis as common metabolic traits,

while the Enteropluri® test provided genus and species-level identifications. *Enterobacter* was the dominant genus among the identified isolates, while nearly half of the isolates remained unidentified. Antibiotic susceptibility testing using the Kirby-Bauer method against 14 antibiotics from 8 classes revealed prevalent resistance to β -lactam antibiotics (amoxicillin, cefazolin, and ampicillin) and sulphamethoxazole, likely linked to their widespread use in human and veterinary medicine. Furthermore, ESBL production was confirmed in 21 isolates, predominantly from the Mooi River (18 isolates) and the Setumo Dam (3 isolates), with key resistance genes (*bla*TEM, *bla*CTX, *Int1*, *AmpC*, *sul1*, and *sul2*) detected via PCR and gel electrophoresis. Importantly, screening for carbapenemase genes (*bla*OXA, *bla*NDM, and *bla*KPC) revealed their presence in selected isolates, raising concerns about future treatment challenges for ESBL-producing pathogens. Sanger sequencing of the 16S rRNA gene identified the *Citrobacter* spp. as the most abundant genus (44%), followed by *Rahnella* spp. (17%), *Enterobacter* spp. (12%), and others. Leveraging on technological advancements such as whole genome sequencing, four genomes were identified from the *Citrobacter* genus: *C. braakii* (S21 and S23), *C. murlinae* (S24), and *C. portucaleensis* (S25). These four genomes demonstrated ANI values exceeding 98%, confirming a close genomic relationship to genomes in the NCBI database. Interestingly, genomic strain S25 was identified as *Citrobacter portucaleensis* by pubMLST and it shared 90% of its genetic make-up with *Citrobacter freundii*, highlighting its close evolutionary association. All four genomes were characterised as ESBL-producing *Citrobacter* species, harbouring the *bla*CMY gene, which is a key determinant of β -lactam antibiotic resistance.

This study also highlighted the virulence genes associated with adhesion, biofilm formation, siderophore production, environmental stress response, and iron acquisition. Genes encoding cell surface localisation components of the capsular polysaccharide system (CPS), such as *vexABCDE*, and capsule polysaccharide biosynthesis genes (*tvfBCDE*) were uniquely identified in the *C. braakii* genomes, providing distinguishing molecular markers for this species. In contrast, the *C. murlinae* strain (S24) exhibited a distinct virulence profile, characterised by the presence of the *iroABCDEF* gene cluster, which encodes the heme receptor protein *shuA*, integral to the iron-salmochelin siderophore system. In addition to the antibiotic resistance observed, these virulence genes and patterns confirm the pathogenetic characteristics of these four genomes. This was supported by results from the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis, which aligned with observations from phenotypic analysis.

This findings highlight the prevalence and diversity of antibiotic-resistant Enterobacteriaceae in surface water systems, highlighting their contribution to water quality degradation and public health risks. The findings demonstrate the urgent need for continuous water quality monitoring through comprehensive epidemiological efforts, targeted wastewater management interventions,

and robust antibiotic resistance mitigation strategies to safeguard human, animal, and environmental health within the One Health framework. Moreover, the presence of antibiotic-resistant Enterobacteriaceae in these water systems poses a significant risk of contamination to crops irrigated directly with this water. This could have far-reaching implications, including reduced food quality, compromised food safety, and potential threats to food security, particularly in vulnerable communities.

Key terms: Enterobacteriaceae, antibiotic resistance, extended-spectrum β -lactamases, surface water quality, virulence factors and whole genome sequencing.

Beginning 12:7

ESBL **One-Health**

MDR **WWTP/Agric/Recreation**

WGS **ARB** **ARG** **Methodology**

Virulence **Pathogen** **Molecular biology**

ENTEROBACTERIACEAE *Water*

Faecal Contamination **Culture-Based** *Characteristics*

LIST OF ABBREVIATIONS

°C:	Degrees Celsius
μ:	Micro
ANI:	Average nucleotide identity
AR:	Antibiotic resistance
ARB:	Antibiotic-resistant bacteria
ARGs:	Antibiotic-resistant genes
BLAST:	Basic Local Alignment Search Tool
bp:	Base pair
CARD:	Comprehensive Antibiotic Resistance Database
CDC:	Centers for Disease Control and Prevention
CFU:	Colony forming unit
CT-SMAC:	Cefoxitin and Potassium tellurite containing Sorbitol MacConkey Agar
DNA:	Deoxyribonucleic acid
DWAF:	Department of Water Affairs and Forestry
DWPF:	Drinking Water Production Facility
DWS:	Department of Water and Sanitation
<i>eaeA</i> :	Attachment and effacing gene (Intimin)
e.g.:	For example (<i>Exempli gratia</i>)
EPA:	Environmental Protection Agency
ESBL:	Extended-spectrum β-lactam
FAO:	Food and Agriculture Organization
FDA:	Food and Drug Administration

g:	Gram
GDP:	Gross domestic product
GLASS:	Global Antimicrobial Resistance Surveillance System
HC:	Haemorrhagic colitis
HGT:	Horizontal gene transfer
<i>HlyA</i> :	Haemolysin A gene
HUS:	Haemolytic uraemic syndrome
ISs:	Insertion sequences
KEGG:	Kyoto Encyclopedia of Genes and Genomes
L:	Litre (s)
LEE:	Locus of enterocyte effacement
mg:	Milligram
mg/L:	Milligram per litre
ml:	Millilitres
MLST:	Multi-locus sequence typing
mm:	Millimetres
MPN:	Most probable number
NCBI:	National Center for Biotechnology Information
NDH:	National Department of Health (RSA)
NDP:	National Development Plan
nm:	nanometres
NMMP:	National Microbial Monitoring Programme
NRF:	National Research Foundation
NWP:	North West province

NWU:	North-West University
OH:	One Health
PCR:	Polymerase chain reaction
RAST:	Rapid Annotation using Subsystem Technology
RSA:	Republic of South Africa
SDG:	Sustainable Development Goal
STEC:	Shiga-toxin-producing <i>Escherichia coli</i>
Stx:	Shiga toxin gene
TTP:	Thrombotic thrombocytopenic purpura
TWQR:	Target Water Quality Range
UN:	United Nations
v/v:	Volume per volume (100ml)
w/v:	Weight per volume (100ml)
WHO:	World Health Organization
WGS:	Whole genome sequencing
WRC:	Water Research Commission
WWTP:	Wastewater Treatment Plant

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CHAPTER 1

INTRODUCTION

1.1 General introduction and problem statement

The United Nations (UN) has set an ambitious agenda to achieve the Sustainable Development Goals (SDGs) by 2030. However, emerging challenges threaten the timely realisation of these goals (Arora et al., 2022; Sachs et al., 2022). SDG 6 highlights the urgent need to safeguard freshwater resources, improve water quality, and effectively manage untreated wastewater (Arora et al., 2022; Messerli et al., 2019). Despite global efforts, the UN and World Health Organisation (WHO) report that 4.2 billion people still lack access to safely managed sanitation, and approximately 80% of the world's wastewater is discharged untreated into the environment (UNICEF, 2019). This has catastrophic public health implications, as the Global Burden of Disease Report (2015) attributes 2,195 child deaths daily to diarrhoea, with nearly 88% of these fatalities linked to unsafe water sources (King et al., 2003). Surface water bodies like rivers and dams are especially vulnerable to these untreated wastewater pollutants (Preisner et al., 2020).

Surface water is a finite resource that is increasingly at risk due to climate-induced pressures such as droughts, floods, and anthropogenic activities (Geris et al., 2022; Bhaga et al., 2020). Projections suggest that by 2025, 1.8 billion people globally will face severe water scarcity, with South Africa expected to be one of the most affected nations (UNICEF, 2019; Ungureanu et al., 2020; Rosegrant et al., 2002). The Department of Water and Sanitation (DWS), in line with the National Water Act (Act No. 36 of 1998), has implemented various mitigation strategies, such as the National Microbial Monitoring Programme (NMMP), to monitor water quality and faecal pollution (van Niekerk et al., 2001; Adeleke et al., 2011). However, significant challenges persist, particularly in the North West province (NWP), where 33 out of 48 wastewater treatment plants (WWTPs) are in critical condition, operating at less than 31% efficiency (DWS, 2023). This inefficiency has led to untreated or poorly treated effluent being discharged into surface water bodies, compounding water quality degradation.

Microbial contaminants, particularly those of faecal origin, significantly threaten water safety. Members of the Enterobacteriaceae family are facultative anaerobic, gram-negative bacteria that are indicators of faecal pollution, thriving in the intestinal tracts of humans and animals (Brenner et al., 2015; Baylis et al., 2011). These organisms, including *Escherichia*, *Enterobacter*, *Serratia*, *Citrobacter*, and *Hafnia*, are commonly called coliforms. Their presence in water signals potential health risks, particularly in surface waters used for drinking, irrigation, and recreational activities

(Edokpayi et al., 2017; Daud et al., 2017). In contrast, *Escherichia coli* (*E. coli*) has been extensively studied as a faecal indicator organism, the ubiquitous non-*E. coli* Enterobacteriaceae, such as *Citrobacter* and *Enterobacter*, remain understudied despite their potential to harbour antibiotic-resistant genes (ARGs) (Croxen et al., 2013; Figueira et al., 2012; Cabral et al., 2010).

Microbes such as the family Enterobacteriaceae are known to have characteristics such as being facultative anaerobes or aerobes, gram-negative rods, able to ferment a wide range of carbohydrates, possess a complex antigenic structure, and produce a variety of toxins and other virulence factors that may pose a water safety concern (Brenner et al., 2015; Baylis et al., 2011). This is because these bacteria thrive in the intestinal tracts of humans and animals, and their presence in water suggests faecal pollution (Manhique et al., 2020). These faecal pollutants, commonly called coliforms, include many genera, such as *Escherichia*, *Enterobacter*, *Serratia*, *Citrobacter*, *Hafnia* and others (Baylis et al., 2011). These coliforms pose potential health concerns and are worth investigating in surface water used for irrigation, drinking water, and recreational activities (Edokpayi et al., 2017; Daud et al., 2017). Particularly of concern are the pathogenic serovars of *E. coli*, such as *E. coli* O157:H7, which are zoonotic in origin and linked to severe diseases, including haemorrhagic colitis and haemolytic uremic syndrome (Rani et al., 2021; McCabe et al., 2019). Livestock, a key reservoir for these pathogens, often serves as a vector for transmission into surface water through agricultural runoff or wastewater discharge (Saeedi et al., 2017; Chase-Topping et al., 2008). Additionally, non-*E. coli* Enterobacteriaceae are increasingly recognised as vectors for the spread of antibiotic resistance (AR), contributing to their clinical and environmental significance (Tesfaye et al., 2019; Pitout et al., 2008).

Emerging contaminants such as antibiotic-resistant bacteria (ARB) and ARGs represent a growing threat to global health, affecting humans, animals, and ecosystems (Feng et al., 2021; Gomes et al., 2020). The One Health framework, adopted as part of the Global Action Plan (2015), highlights the need for a holistic approach to address the AR crisis (WHO, 2019). In South Africa, the National Action Plan prioritises enhanced antibiotic surveillance, particularly for β -lactam and carbapenem-resistant pathogens, to combat resistance (Engler et al., 2020; TOP, 2015). Notably, extended-spectrum beta-lactamase (ESBL) and carbapenem-resistant Enterobacteriaceae (CRE) are emerging as critical global threats due to their ability to inactivate broad-spectrum antibiotics, leading to severe healthcare-associated infections with high mortality rates (Kimera et al., 2021; Shaikh et al., 2015). Sub-Saharan Africa reports an exceptionally high prevalence of ESBL-producing Enterobacteriaceae, yet significant knowledge gaps persist in understanding the prevalence, virulence, and resistance profiles of these organisms in surface water systems (Tesfaye et al., 2019; Sangare et al., 2017).

This study addresses the critical gap in research by investigating the prevalence, pathogenicity, and AR profiles of faecal and ubiquitous Enterobacteriaceae isolated from surface waters in the NWP. Leveraging whole genome sequencing (WGS), this research provides insight into these isolates' genomic characteristics, virulence factors, and resistance determinants. By bridging the knowledge gap on Enterobacteriaceae in South African (RSA) surface waters, this study contributes to advancing water safety, public health, and environmental sustainability, particularly concerning the One Health (OH) framework.

1.2.1 Research aim and objectives

1.2.1 Research aim

This research aims to isolate, characterise and profile Enterobacteriaceae for antibiotic susceptibility and detect (ARGs) using phenotypic and genotypic approaches.

1.2.2 Objectives of this study

The specific objectives of this research were to:

- 1) quantify coliforms in samples from the selected surface waters,
- 2) isolate, characterise and identify pathogenic Enterobacteriaceae from among the coliforms and screen bacterial isolates recovered for extracellular and ESBL enzyme production ability,
- 3) screen bacterial isolates recovered from water samples for antibiotic susceptibility patterns, virulence, ESBL, carbapenems and ARGs,
- 4) unravel chromosomal genetic elements harbouring virulence and ARGs in bacterial isolates recovered from water samples using WGS technology.

CHAPTER 2

LITERATURE REVIEW

2.1 A freshwater perspective

The Water Research Commission (WRC) has long recognised freshwater scarcity as a critical global risk. In its 2017 report, the WRC highlighted water shortages' profound local and global effects. Recent evaluations, including the World Resources Institute's Aqueduct Water Risk Atlas and the 2023 report of the Global Commission on the Economics of Water, reaffirm these concerns, stressing the urgency of addressing this escalating crisis (Grafton et al., 2023). The freshwater crisis is further intensified by declining water quality, the emergence of novel pollutants, and the cumulative effects of anthropogenic activities on already vulnerable water sources (DWAF, 2013). Although freshwater constitutes only 3% of the Earth's total water resources, much of it is locked in glaciers and ice caps, with the remainder distributed among lakes, rivers, reservoirs, wetlands, and groundwater systems. These vital water systems sustain life by serving as a source of drinking water and supporting agriculture through irrigation. They also play critical roles in providing food, energy production through hydropower, and transportation. Despite their indispensable contributions to human and ecological well-being, freshwater habitats are disappearing at an alarming rate, driven by human activities and climate change (WWF, 2023).

Freshwater systems face significant and multifaceted threats. While the global freshwater supply is theoretically sufficient, its uneven spatial and temporal distribution presents considerable challenges (Cosgrove, 2015). Clean freshwater is essential for economic productivity, but its extraction often disrupts ecosystems and biodiversity, imposing significant yet unquantified ecological costs (Downing, 2021; Vörösmarty, 2010). The lack of adequate freshwater resources in many regions constrains human health, productivity, and the ability to achieve environmental sustainability (Koehler et al., 2008). Research indicates that freshwater systems near residential, agricultural, and industrial zones experience heightened disruption in microbial communities and physicochemical characteristics (Vignale, 2023).

Surface water, which serves as a critical source of freshwater for drinking, household activities, and agricultural use (Figueira, 2012; Ivone, 2013), is more than a resource; it is a lifeline. However, its availability is increasingly constrained by climate change, contamination, and population growth (Singh, 2020). Human activities such as habitat destruction, pollution, water over-extraction, and overfishing have driven extreme declines in freshwater biodiversity, with species in freshwater systems now facing a higher risk of extinction than their marine counterparts

(Strayer, 2010; Dudgeon, 2006). Beyond biodiversity, freshwater ecosystems play critical roles in regulating biogeochemical cycles, facilitating carbon sequestration, and influencing greenhouse gas emissions, all essential for maintaining ecological balance and supporting human survival. Anthropogenic climate change disrupts these processes, triggering feedback loops with wide-reaching implications (Torn & Harte, 2006; Cox et al., 2000).

Future challenges to freshwater systems will stem from climate change and other anthropogenic pressures, including population growth, economic development, and technological advancements (Wagenaar, 2018). These factors will influence water availability quantity, quality, and timing, with regional and ecosystem-specific variations determined by their relative vulnerability and resilience (Black, 2010; Leisner, 2020). By 2050, global water demand is projected to increase substantially, exacerbating existing disparities in water access across continents. With the world population expected to rise to 9.4 to 10.2 billion, freshwater resources will face mounting stress, particularly in regions already experiencing chronic water scarcity (Boretti, 2019). Climate change will amplify these challenges, reducing precipitation levels and intensifying evaporation rates in arid areas, further threatening water supplies for large segments of the global population.

2.2 Overview of the freshwater systems in South Africa

South Africa is the 30th most water-scarce country globally (Kohler, 2016). The nation's semi-arid landscape receives an average annual rainfall of 450 mm, significantly lower than the global average of 860 mm, coupled with high evaporation rates from surface water (DWS, 2015). Freshwater resources in South Africa are allocated primarily to food production (60%), followed by domestic use (27%), afforestation (3%), industrial use (3%), livestock watering (2.5%), mining activities (2.5%), and energy generation (2%). However, these vital water resources are increasingly compromised by faecal pollution, rendering them unsuitable for direct use in agriculture, livestock watering, and recreational activities.

2.2.1 Water quality and quantity

The rapid growth of human populations, combined with economic and industrial development, has led to significant deterioration of natural freshwater systems, affecting water quality, biodiversity, in-stream processes, and watershed hydrological regimes (Martinuzzi et al., 2014; O'Driscoll et al., 2010). A study by Jordaan et al. (2016) on the Mooi River system in South Africa highlights the adverse effects of urbanisation on water quality. The findings revealed that urban development degrades water quality and alters the bacterial community composition in these freshwater systems. This highlights the urgency of addressing the challenges posed by urbanisation to preserve both water quality and ecosystem functionality.

2.2.2 Water and land use

The image below (Figure 2.1) illustrates how the quality and quantity of surface water systems are affected by numerous land-use activities.

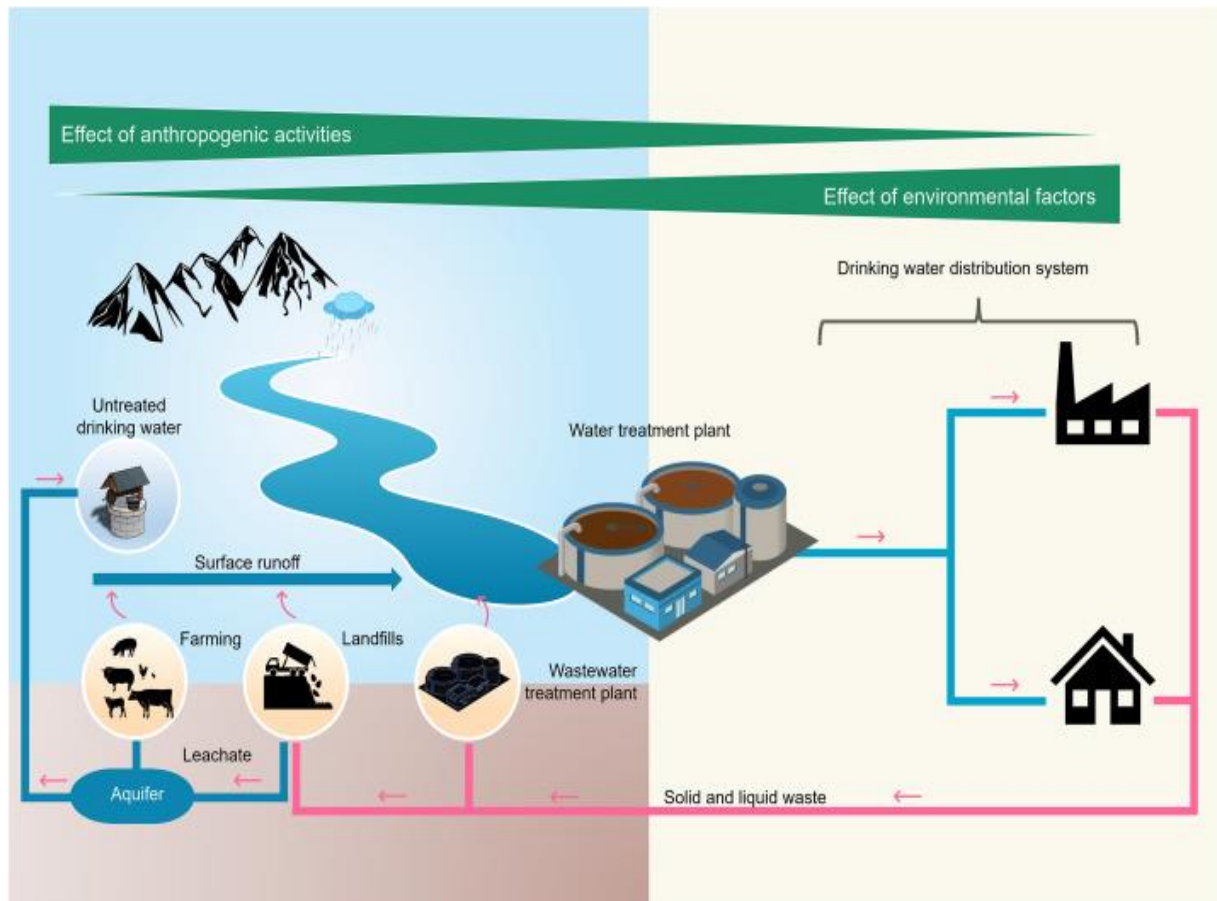


Figure 2.1: Land-use activities that affect the quality and quantity of surface water systems.

Accessed: (Sanganyado and Gwenzi, 2019) on 14 September 2022.

Freshwater systems are increasingly exposed to pollutants from anthropogenic activities such as municipal, industrial, mining and agricultural operations, and WWTPs. These activities discharge a wide range of organic and inorganic contaminants into surface water systems, including nutrient loads, heavy metals, and biological materials (Jordaan et al., 2019; Ford et al., 2018). Such disturbances significantly impair these habitats' ecological structure and function (Paerl et al., 2003). The compromised water quality often renders it unfit for recreational use or other human purposes, as illustrated in Figure 2.1, posing substantial public health risks (De Figueiredo et al., 2010).

2.2.3 Waterborne diseases

Contaminated freshwater is a significant vector for the transmission of waterborne diseases, including dysentery, cholera, diarrhoea, and typhoid, which continue to cause substantial morbidity and mortality globally, particularly in developing countries (Niza-Ribeiro et al., 2022). The WHO reports that over 80% of diseases worldwide are linked to contaminated water and waterborne pathogens (Haseena et al., 2017). Despite being largely preventable, waterborne diseases claim 842,000 lives annually, with young children in developing countries bearing the most significant burden (WHO, 2018; Ford et al., 2010). Ensuring universal access to clean water and sanitation, as outlined in SDG 6, is a critical strategy to reduce the prevalence of these diseases (Griffiths et al., 2017).

Poor waste management, a lack of handwashing, bodily cleaning, and limited access to clean water exacerbate the problem, making sanitation and hygiene crucial for disease prevention. The ingestion of contaminated water, combined with inappropriate separation of human and animal faeces from food sources, contributes significantly to the spread of waterborne diseases (Szálkai et al., 2023). Pathogenic bacteria, including *Salmonella*, *Shigella*, *Escherichia coli*, and *Campylobacter*, are among the most prominent causes of these diseases (Tanner et al., 2019). Moreover, waterborne pathogens can serve as reservoirs for AR, further complicating treatment and containment efforts (Cho et al., 2023; Cohen et al., 2020).

2.2.4 Infection potential

Bacterial infections occur when microorganisms invade and proliferate in a host, often causing disease or illness (Monack et al., 2004). These infections typically involve colonisation, invasion, proliferation, immune response, and pathogenesis, frequently resulting in chronic conditions or recurrent infections (Rock et al., 2014). Specific strains, such as *E. coli* O157, possess virulence factors like the intimin gene, facilitating adherence to epithelial cells via the locus of enterocyte effacement (LEE) pathogenic island (Harrington et al., 2006; Kaper, 2004). Similarly, related bacteria such as *Citrobacter rodentium* exhibit comparable mechanisms of pathogenesis.

The emergence of antibiotic-resistant pathogens exacerbates the role of bacterial infections in human health. Enterobacteriaceae species are particularly concerning, as they include both community-acquired and nosocomial infections, with rising resistance to critical antibiotics like β -lactams and carbapenems (Navidinia et al., 2016). Surveillance and promptly identifying resistant strains are crucial in mitigating their spread (Wallace et al., 2020). Additionally, the pathogenesis of Shiga toxin-producing strains (STEC) of *E. coli* O157 highlights the need for targeted research and intervention strategies to prevent outbreaks (Nataro et al., 2011; Iredell et al., 2016).

2.2.5 Selected surface water sources

2.2.5.1 Mooi River

The Mooi River catchment, covering approximately 1,800 km², spans parts of western Gauteng and the North West Province of South Africa and serves as the primary water supply for Potchefstroom in the North West Province. Potchefstroom has a population of approximately 148,804 residents (City Population Census, 2011). The catchment is fed primarily by dolomitic eyes and springs, contributing to its flow (Van der Walt et al., 2002). However, the water quality of the Mooi River and its tributaries, such as the Wonderfontein and Loop Spruit, has been influenced significantly by human activities, including agriculture (covering over 20% of the land area), urbanisation, and informal settlements (Bosch et al., 2023; Van der Walt et al., 2002).

The river originates in the Boons area north of Potchefstroom and flows southwards into the Klerkskraal Dam, passing through Potchefstroom before eventually joining the Vaal River (Malherbe et al., 2020). The upper section of the river, from its origin to the Klerkskraal Dam, remains in excellent condition due to limited land use impacts (Koekemoer et al., 2021; Le Roux et al., 2005). However, land use activities in surrounding areas, such as mining and agriculture, have caused deterioration of the water quality downstream, particularly in the tributaries (Van der Walt et al., 2002; Bredenhann et al., 2022; Jordaan et al., 2019). Several studies have documented these effects on the river's water chemistry and biota, highlighting its vulnerability to human-induced changes (Bosch et al., 2023; Wanda et al., 2017; Barnard et al., 2013).

2.2.5.2 Schoonspruit River

The Schoonspruit River, a tributary of the Vaal River, is located in the NWP and spans a catchment area of 325 km² (Tooth et al., 2004). Situated in a sub-humid, warm region with wet summers and cool, dry winters, its terrain elevation is approximately 1,289 metres above sea level. This water system primarily supplies Ventersdorp and Klerksdorp. According to the City Population (2011), 22,073 and 189,496 people are being served, respectively. The Schoonspruit River is part of the Middle Vaal Water Management Area (WMA) and is one of this region's five major rivers. Wetland habitats characterise the river's upper reaches, while its middle sections feature a narrow channel dominated by willows and white poplars below Ventersdorp. In the lower reaches, the Schoonspruit transitions into an extensive wetland system (DWAF, 2007).

The river flows over erodible Karoo Supergroup shales in its upper and middle sections, crossing a resistant dolerite sill downstream. Its water supply is enhanced by dolomite springs in the upper catchment, which provide irrigation and urban water for Ventersdorp (DWAF, 2004). However,

diamond-digging operations along the riverbanks have introduced significant pollution, affecting water quality and sediment composition downstream (Lusilao-Makiese et al., 2014; WRC, 1998). Additionally, the interaction between groundwater and surface water in the dolomitic compartments contributes an estimated 26.7 million m³ of water annually, along with associated salt loadings, to the Vaal River (Midgley et al., 1994). The construction of numerous small earth dams along its channels and tributaries has further altered the Schoonspruit's flow regime, reducing its mean annual runoff to 20–50 mm (Tooth et al., 2004). Mining activities have also contaminated the bottom sediments of reservoirs downstream of the Schoonspruit, highlighting the severe ecological impacts of anthropogenic activities on the river system (Lusilao-Makiese et al., 2014; DWAF, 2004).

2.2.5.3 Setumo Dam

The Setumo Dam is a critical raw water source for municipal use, domestic purposes, and livestock watering in the Mahikeng area, located in the Ngaka Modiri Molema District Municipality of the NWP. This dam is also a popular fishing site for local communities (Ndou et al., 2023). However, recent years have seen a significant decline in water quality, with severe ecological consequences, including fish morbidity that left numerous dead fish washed up along the dam's banks (Munyati et al., 2015).

Mahikeng, with a population of approximately 354,504 people, is predominantly urbanised, with over 80% of its residents living in urban areas (City Population Census, 2022). The increasing human population and urbanisation have intensified pressure on the region's natural resources, including water (Akoth, 2018). This pressure has led to a higher yield of wastewater, requiring treatment by the area's two WWTPs. However, these plants are often inadequate, resulting in insufficiently treated wastewater being discharged into the Setumo Dam. This has raised concerns over faecal contamination and its potential effect on water quality and public health (Juma et al., 2014).

2.3 Coliforms as microbial water quality indicators

Coliform bacteria belong to the Enterobacteriaceae family, although not all Enterobacteriaceae are classified as coliforms. Coliforms are naturally present in the digestive tracts of animals, including humans, and in their waste products. They are also commonly found in plant material and soil (Janda et al., 2021; Farmer et al., 2010). The coliform group, which includes species such as *Escherichia*, *Citrobacter*, and *Enterobacter*, has long been used as a microbial indicator to assess faecal contamination in various water systems, including surface water, recreational water, and drinking water. The resilience of coliform bacteria compared to pathogenic

microorganisms makes them an effective indicator of water quality. Their absence from water suggests that it is bacteriologically safe for human consumption. Conversely, their presence implies that other potentially pathogenic microorganisms might also be present, rendering the water unsafe to drink (Walker et al., 2019; Gerba et al., 2009). Consequently, coliforms serve as a critical early warning system for identifying potential public health risks associated with contaminated water.

Total coliforms are widely distributed in the aquatic environment, soil, vegetation, and the intestines of mammals, including humans (Cooney et al., 2014; An et al., 2002). Despite ongoing debates about their reliability as indicators of faecal contamination, total coliforms remain a widely used regulatory parameter in many countries (Ivone et al., 2013; Cooney et al., 2014; An et al., 2002). This continued use is attributed to their ability to act as a sensitive indicator, given that they are often more numerous than *E. coli*, even in faecal contamination (Maheux et al., 2014; An et al., 2002). Several environmental factors influence the decay rates of faecal coliform bacteria in aquatic environments. Higher temperatures accelerate bacterial die-off, while elevated pH levels and increased dissolved oxygen levels negatively affect bacterial survival. Solar radiation, particularly ultraviolet rays, inhibits bacterial persistence, and predatory microorganisms, such as protozoa, further reduce coliform populations. Additionally, limited nutrient availability and high salinity contribute to faster bacterial die-off (An et al., 2002). These environmental conditions collectively determine the survival and persistence of faecal coliforms, influencing their reliability as water quality indicators.

Historically, coliforms were not considered a significant threat to human health because they are typically non-pathogenic. However, coliform bacteria have emerged as pathogens in recent years, particularly in immunocompromised individuals and hospital environments. Their ability to cause infections under such circumstances has drawn increased attention to their role in human health (Jesudason, 2024). Coliform bacteria, particularly total coliforms, remain indispensable microbial indicators to monitor water quality. Their presence highlights potential contamination risks and informs regulatory actions to protect public health (Yates, 2007). While environmental factors influence their survival, the increasing recognition of coliforms as emerging pathogens necessitates continuous surveillance, particularly in vulnerable populations and healthcare settings (Lépesová et al., 2019; Dhiraputra et al., 2005). Using coliforms as indicator organisms for faecal contamination highlights the importance of microbial water quality monitoring in safeguarding both environmental and public health (Lépesová et al., 2019; Invik et al., 2017; Dhiraputra et al., 2005).

2.4 Characteristics of Enterobacteriaceae

Members of the Enterobacteriaceae family are highly adaptable, capable of thriving in diverse environments while being isolated from a wide range of host species across various biological kingdoms (Neil et al., 2023; Neil et al., 2018). High concentrations of coliform bacteria, including Enterobacteriaceae, have been detected in lakes and drinking water reservoirs, particularly during the summer when favourable conditions for bacterial growth prevail (Leister et al., 2022; Reitter et al., 2021). These bacteria frequently contaminate soil and freshwater sources via sewage, animal faeces, manure, agricultural runoff, and other anthropogenic pollution (Walker et al., 2019). In the NWP of South Africa, Enterobacteriaceae have been implicated in the dissemination of ARGs in aquatic environments and the transmission of waterborne diseases (Makuwa et al., 2023; Ateba et al., 2020; Mulamattathil et al., 2014).

The Enterobacteriaceae family is a heterogeneous group of gram-negative, rod-shaped, non-sporulating bacteria belonging to the class Gamma Proteobacteria. They are facultative anaerobes with simple nutritional requirements and metabolic versatility, including fermenting glucose and reducing nitrate to nitrite. Morphologically, they measure 2 to 4 µm in length and 0.4 to 0.6 µm in width, possess rounded ends, and have an in vitro generation time of approximately 20 to 30 minutes (Morales-López et al., 2019; Janda et al., 2021; Farmer et al., 2010). While some members are non-motile, motile species typically use peritrichous flagella for movement. Their cell walls share features standard to gram-negative bacteria, including a peptidoglycan layer, inner and outer membranes, and a periplasmic space (Moxley et al., 2022; Janda et al., 2021).

The Enterobacteriaceae family includes over 40 genera and 150 named species, with a growing number of subspecies, biogroups, and DNA hybridisation groups (Farmer et al., 2010; Janda et al., 2021). Members such as *Escherichia*, *Citrobacter*, *Klebsiella*, *Salmonella*, *Shigella*, and *Yersinia* have immense public health importance due to their role in diseases like pneumonia, diarrhoea, gastroenteritis, urinary tract infections, and sepsis (Fadare et al., 2020; Cooney et al., 2014). Beyond clinical settings, the family also includes key foodborne pathogens, such as *Salmonella*, *Escherichia coli*, *Shigella*, and *Yersinia enterocolitica*, which are significant contributors to global disease burdens (Cooney et al., 2014; Ivone et al., 2013). Various molecular techniques have been employed to characterise Enterobacteriaceae strains, including serotyping, plasmid profiling, ribotyping, and next-generation sequencing (NGS) (Chenhaka et al., 2023; Cooney et al., 2014; Ivone et al., 2013).

2.4.1 *Escherichia coli*

The genus *Escherichia* was named after Theodor Escherich, who first isolated *Escherichia coli* from faeces in 1885. This gram-negative, rod-shaped, facultatively anaerobic bacterium is a prominent member of the Enterobacteriaceae family and resides in the intestines of warm-blooded animals (Janda et al., 2021; Farmer et al., 2010). Measuring 1.1 to 1.5 µm wide and 2 to 6 µm long, *E. coli* cells can be motile or non-motile, with motile strains using peritrichous flagella for movement (WHO, 2018; Farmer et al., 2010). Many strains also produce additional surface structures, such as fimbriae or pili, which mediate attachment to host tissues and surfaces.

E. coli serotyping is based on combinations of O (lipopolysaccharide), H (flagella), and K (capsular polysaccharide) antigens. Currently, 181 O antigens, 53 H types, and 80 K antigens are recognised, with serotypes such as *E. coli* O157:H7 being epidemiologically significant (WHO, 2018; Farmer et al., 2010). Most strains are commensal and harmless, but pathogenic variants cause various diseases. Pathogenic strains are categorised into six groups based on their virulence properties, including enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enteroaggregative (EAEC), diffusely adherent (DAEC), and STEC strains (Estrada-Garcia et al., 2014; Van Elsas et al., 1998).

Pathogenic *E. coli* strains are primarily transmitted through contaminated food and water and are associated with gastrointestinal illnesses such as diarrhoea, haemorrhagic colitis, and haemolytic uremic syndrome (HUS) (Yang et al., 2014; Estrada-Garcia et al., 2014). Extraintestinal infections, including urinary tract infections (UTIs) and neonatal meningitis, are also common (Estrada-Garcia et al., 2014; Farmer et al., 2010). The high mobility of virulence genes in *E. coli*, especially in enterohemorrhagic strains, raises concerns about the emergence of new pathotypes (Yang et al., 2014; Desmarchelier et al., 2011). Due to its easy genetic manipulation, *E. coli* remains a model organism in biotechnology, widely used in recombinant protein production and synthetic biology (Eastwood et al., 2023; Pontrelli et al., 2018).

Due to its long history of laboratory culture and ease of manipulation, *E. coli* plays an important role in modern biological engineering and industrial microbiology (Pontrelli et al., 2018; Russo et al., 2003; Cornelis et al., 2000; Lee et al., 1996). *E. coli* is a very versatile host to produce heterologous proteins (Meng et al., 2020; Pontrelli et al., 2018) and various protein expression systems have been developed that allow the production of recombinant proteins in *E. coli* (Eastwood et al., 2023). Some attempts have also been made to engineer *E. coli* for the efficient production of beneficial compounds (Ferreira et al., 2020; Rosano et al., 2019; Kumar et al., 2020), e.g., butanol, squalene, glycoprotein vaccines.

2.4.2 *Citrobacter* spp.

Species in the genus *Citrobacter* are facultatively anaerobic, gram-negative bacilli motile via peritrichous flagella. They are commonly isolated from water, soil, food, and the intestines of humans and animals (Pilar et al., 2020; Ranjan et al., 2013). The genus *Citrobacter* was first described in 1932 and includes at least 11 species, including *C. freundii*, *C. koseri*, and *C. braakii* as the most clinically relevant (Brenner et al., 1993). Although primarily opportunistic pathogens, *Citrobacter* species have been implicated in nosocomial infections, including urinary tract infections, pneumonia, and septicemia, particularly in immunocompromised patients (Vignale et al., 2023; Yang et al., 2018).

Citrobacter freundii and *C. koseri* are frequently isolated from infections and are known to cause meningitis, septicemia, and pulmonary diseases, especially in neonates and individuals with predisposing conditions (Yang et al., 2018; Metri et al., 2013). On MacConkey (MAC) agar, *Citrobacter* species can ferment lactose, producing colonies that resemble *E. coli*. Despite their similarities to *Salmonella*, *Citrobacter* species are distinguished by their slower urease hydrolysis and environmental ubiquity (Chart et al., 2012).

2.4.3 *Enterobacter* spp.

The genus *Enterobacter* was first proposed by Hormaeche and Edwards in 1960 (Cooney et al., 2014; Chart et al., 2012). The species are widely dispersed in nature and exist in diverse environments, including soil, water, domestic and food processing establishments, vegetation, vertebrate and invertebrate hosts, and in the faeces of humans and animals. The bacteria grow aerobically and anaerobically at temperatures between 20 and 37°C on general laboratory media at neutral pH. It has variable motility (Farmer et al., 2010; Janda et al., 2021).

Most *Enterobacter*-associated infections are hospital-acquired. These bacteria contaminate hospital surfaces, medical equipment, and personnel. *Enterobacter* is a member of the ESKAPE group, which contains the major resistant bacterial pathogens. The members of this group have proven to be more complex due to the exponential evolution of phenotypic and genotypic methods. However, as of today, 22 species belong to the *Enterobacter* genus (Davin-Regli et al., 2019). Little is known about the pathogenicity and virulence factors of *Enterobacter* spp. Due to the paucity of studies in this area (Davin-Regli et al., 2019).

2.4.4 *Hafnia* spp.

The genus *Hafnia* currently consists of two recognised species, *Hafnia alvei* and *Hafnia paralvei* (Pan et al., 2022; Huys et al., 2010). These species have been isolated from diverse environmental and clinical sources, including soil, water, and food products such as minced beef, vacuum-packed beef, pork products, dairy products, freshwater fish, and honey (Merchán et al., 2022; Janda et al., 2006). *Hafnia* spp. are facultatively anaerobic, gram-negative bacilli that do not ferment lactose, making them resemble *Salmonella* when grown on specific *Salmonella* isolation media (Cooney et al., 2014). Despite this, no particular culture medium has been developed exclusively for detecting *Hafnia* spp.

Biochemically, *Hafnia* spp. exhibit reactions like those of *Enterobacter* and *Serratia*. Their role in human infections remains poorly understood. While most strains are not pathogenic to healthy individuals, *Hafnia* spp. act as opportunistic pathogens in immunocompromised individuals, particularly those who are elderly, very young, pregnant, or suffering from severe underlying illnesses such as hematologic malignancies, pulmonary diseases, liver cirrhosis, or pancreatitis (Patel et al., 2014). Infections caused by *Hafnia* spp. are rare, typically involving faecal isolates, with limited recovery from other body sites.

Hafnia spp. exhibit variable antimicrobial resistance profiles (Rizi et al., 2021; Jayol et al., 2017). However, limited information is available regarding their virulence mechanisms. Hypothesised mechanisms include the production of siderophores, type 1 fimbriae (mannose-sensitive hemagglutination), type 3 fimbriae (mannose-resistant *Klebsiella*-like hemagglutination), lipopolysaccharide (LPS), and their ability to invade epithelial cells and resist human serum bactericidal action (Smith et al., 2014). One notable feature of *Hafnia alvei* is its production of acyl-homoserine lactones (AHLs), quorum-sensing signalling molecules that regulate enzymatic activity, particularly in fermented foods where this bacterium is frequently found (Ramos-Vivas et al., 2022).

2.4.5 *Serratia* spp.

The genus *Serratia* comprises motile, non-endospore-forming, gram-negative rods that belong to the Enterobacteriaceae family (Barman et al., 2020; Kämpfer et al., 2015). Currently, 17 species in this genus are well-characterised, including *S. marcescens*, *S. plymuthica*, *S. liquefaciens*, *S. rubidaea*, *S. fonticola*, and *S. proteamaculans* (Fusco et al., 2018; Cooney et al., 2014). These organisms are commonly found in soil, water, plants, insects, and even air samples. They are opportunistic pathogens that can cause infections in hospital settings and in immunocompromised individuals.

Serratia spp. are notable for producing a wide array of virulence factors, including DNase, lipase, gelatinase, haemolysin, proteases, and chitinase, as well as secondary metabolites such as prodigiosin (a red pigment) and biosurfactants (Fusco et al., 2018; Ishii et al., 2014). The pigment is particularly characteristic of *S. marcescens*, differentiating it from other Enterobacteriaceae when grown on nutrient agar (Farmer et al., 2010). Pigmented strains are occasionally associated with respiratory tract and faecal samples. However, red-pigmented strains can also be misinterpreted as blood contamination in food or clinical samples (Golemi-Kotra et al., 2008).

Infections caused by *Serratia* spp. are predominantly nosocomial (hospital-acquired), affecting the urinary tract, respiratory system, bloodstream, and wounds (Bourdin et al., 2023; Montagnani et al., 2015). The rise of multidrug-resistant (MDR) strains, particularly in neonatal intensive care units, is an increasing concern. Outbreaks have been linked to contaminated transfusion fluids and hospital equipment, emphasising the critical need for robust infection control measures (Li et al., 2022; Matsushita et al., 2009).

2.4.6 *Klebsiella* spp.

The genus *Klebsiella*, a significant member of the Enterobacteriaceae family, consists of gram-negative, non-motile, encapsulated, rod-shaped bacilli that ferment lactose. These bacteria are commonly found in human skin, mucous membranes, soil, freshwater, and even saltwater environments (Azam et al., 2023; Paczosa et al., 2016). The genus was first described in the 19th century and was historically called "Friedländer's bacterium" (Merino et al., 1992). Advances in molecular taxonomy have since divided *Klebsiella* into five species and three related genera, including the *Raoultella* species (Barson and Leber, 2018).

Klebsiella pneumoniae, the most clinically relevant species, is divided into three subspecies: *pneumoniae*, *ozaenae*, and *rhinoscleromatis*. It is an opportunistic pathogen known for causing urinary tract infections (UTIs), septicaemia, pneumonia, and neonatal meningitis (Azam et al., 2023). The bacterium's pathogenicity is attributed to its virulence factors, including the production of capsular polysaccharides that inhibit phagocytosis, haemolysin, and iron-chelating compounds, enabling it to evade immune responses and colonise host tissues effectively.

2.4.7 *Rahnella* spp.

The genus *Rahnella* in the Enterobacteriaceae family consists of gram-negative, facultatively anaerobic rods that exhibit motility through peritrichous flagella (Watson et al., 2018; Kämpfer et al., 2015). *Rahnella* spp. can grow at low temperatures (as low as 4°C) and demonstrate typical metabolic traits such as nitrate reduction, catalase positivity, and oxidase negativity. Phylogenetic

studies show high similarity between *Rahnella aquatilis*, *Hafnia alvei*, and *Yersinia enterocolitica* (Katalin et al., 2012).

Rahnella is most prevalent in freshwater systems, but the genus is also found in soil, plant rhizospheres, and the intestines of some invertebrates. While it is typically non-pathogenic, *Rahnella* spp. have been isolated from cases of gastroenteritis, bacteraemia, and septicemia, particularly in immunocompromised individuals. This genus produces β -lactamases, though infections are generally treated with extended-spectrum cephalosporins, aminoglycosides, or fluoroquinolones (Watson et al., 2018).

2.5 Antibiotic resistance

Antibiotics are pivotal in combating diseases across human, terrestrial, aquatic systems, and agricultural landscapes. However, their efficacy is diminishing due to the rising prevalence of AR. According to the Food and Agriculture Organization (FAO) and the WHO, this global crisis is primarily driven by the misuse and overuse of antibiotics in human medicine, veterinary practices, and agriculture, accelerating the development and dissemination of resistance (WHO, 2022; Canton, 2021; Frieri et al., 2017). A surveillance report by the National Department of Health (NDH) in the RSA (2021) highlighted that approximately 60% of all procured antimicrobials were used in food production, while 40% were allocated to treating human infections. Commonly used antibiotics included extended-spectrum penicillins and trimethoprim-sulphamethoxazole for human diseases, while penicillins, tetracyclines, sulphonamides, and macrolides were frequently used in agricultural settings (NDH, 2021).

AR has been reported at alarming levels across countries with varying income levels. This poses significant challenges, as diseases that were once easily treatable are now becoming untreatable, and life-saving medical interventions are increasingly risky (Podolsky et al., 2018; Rai et al., 2016). Resistance is escalating rapidly among diverse microorganisms, including bacteria, fungi, viruses, and protozoa (Frieri et al., 2017; Rai et al., 2016). The implications of this trend extend beyond public health, with drug-resistant infections emerging as a global threat that has the potential to become one of the leading causes of morbidity and mortality (Jasovský et al., 2016). Furthermore, AR could exacerbate extreme poverty, hunger, and malnutrition, while causing severe economic losses, projected to erode several per cent of the global gross domestic product (GDP) (Canton, 2021).

Resistance in bacteria can be categorised as intrinsic or acquired. Intrinsic resistance is inherent to particular bacterial species, meaning all members of that species naturally exhibit this resistance pattern. In contrast, acquired resistance arises through mutations in existing DNA or

the acquisition of ARGs via horizontal gene transfer (HGT) (Darby et al., 2023; Blair et al., 2015; Frieri et al., 2017). ARGs are often located in genetic contexts with limited mobility. However, some have gained increased mobility potential through association with small transposable elements, such as insertion sequences (ISs), enabling their dissemination across bacterial populations (Berglund et al., 2023; Frieri et al., 2017).

Understanding the resistance mechanisms is crucial for developing strategies to mitigate their global impact. The resistance mechanisms reflected in Table 2.1 include the enzymatic degradation of antibiotics, chemical modification of antibiotic molecules, target site alteration or bypass, and efflux mechanisms that expel antibiotics from bacterial cells (Smith et al., 2023; Darby et al., 2023; Blair et al., 2015). These molecular strategies enable bacteria to evade the action of antimicrobials, rendering treatments ineffective. Research on these mechanisms informs the rational use of existing antibiotics, the design of novel drugs less prone to resistance, and innovative approaches to combat resistance (Smith et al., 2023; Darby et al., 2023). AR threatens to undermine decades of medical progress without decisive intervention, with dire consequences for human, animal, and environmental health.

2.5.1 Surface water's role in the spread and persistence of antibiotic resistance

The everyday use of antibiotics across various sectors, as illustrated in Figure 2.2, has improved the quality of life and aided in economic growth; however, due to the bioaccumulation of these therapeutic agents in the environment, microbes are developing resistance.



Figure 2.2: An illustration of the effect of antibiotic use on surface water systems.

(Adapted from Zhang et al., 2020; Dillard et al., 2022).

Surface water serves as a critical reservoir and vector for the dissemination and persistence of AR. It provides a medium where ARB and ARGs proliferate and disperse via complex environmental pathways (Magome, 2021). Increasing evidence highlights that surface water bodies, including rivers, lakes, and streams, frequently receive input from agricultural runoff, WWTP effluents, and industrial discharges, all of which can harbour substantial loads of ARB and ARGs (Kraemer et al., 2020; Manaia et al., 2018; Marti et al., 2014). These anthropogenic contributions create reservoirs of resistance in surface waters, where bacteria persist and multiply, particularly in nutrient-rich environments that favour bacterial growth (Rizzo et al., 2013).

The HGT process facilitates the exchange of genetic material, including ARGs, which is particularly active in aquatic environments. HGT allows resistance genes to spread in bacterial populations and across different species, thereby expanding the resistance pool (Huijbers et al., 2015; Baquero et al., 2008). Biofilm formation in aquatic systems further promotes bacterial survival and facilitates HGT, providing a protective matrix that enhances resistance gene exchange and shields bacteria from environmental stressors (Sharma et al., 2016; Amos et al., 2014).

One of the primary contributors to resistance dissemination is the discharge of inadequately treated wastewater from WWTPs. Studies have shown that WWTPs often fail to completely remove antibiotics, ARB, or ARGs from treated effluent, releasing them into receiving water bodies (Guo et al., 2018; Manaia, 2017; Michael et al., 2013). Downstream of WWTP discharge points, these effluents create "hotspots" of resistance, amplifying the local concentration of ARB and ARGs and enabling their proliferation (Guo et al., 2018). Similarly, agricultural runoff, especially from fields treated with manure or livestock waste as fertiliser, introduces antibiotics and resistant bacteria into nearby surface waters, compounding the persistence of resistance in aquatic environments (Kraemer et al., 2020; Topp et al., 2018).

The impact of surface water contamination extends beyond environmental health as resistance spreads through the food chain. Irrigation with contaminated water can introduce ARB and ARGs to crops, which humans consume (Bennett & Mentzel, 2020). Additionally, aquatic animals such as fish exposed to resistant bacteria in contaminated water can serve as reservoirs of resistance, posing further risks when consumed (Cabello et al., 2016). Environmental factors such as temperature, pH, and nutrient availability influence the survival and persistence of resistant bacteria in aquatic systems, while hydrological events such as heavy rainfall and seasonal flooding mobilise ARGs and ARB from sediments and soils into water bodies (Sabri et al., 2020; Finley et al., 2013).

Urbanisation and intensified agricultural practices exacerbate the accumulation of ARGs in surface waters, increasing human exposure risks, particularly in regions with limited wastewater management infrastructure (Sabri et al., 2020). Recreational activities in contaminated water systems further contribute to public health risks, as individuals exposed to resistant bacteria may become colonised, with vulnerable populations especially at risk (Leonard et al., 2015). The global interconnectedness of waterways complicates containment, as rivers, lakes, and streams act as transboundary conduits, disseminating ARGs and ARB across regions and countries (Wellington et al., 2013; Kümmerer, 2009). Addressing the role of surface water in AR requires an integrated OH approach, which recognises the interconnectedness of human, animal, and environmental health.

2.5.2 The One Health framework

The OH approach is a collaborative, transdisciplinary framework that aims to safeguard and promote the health of humans, animals, and environmental health by integrating surveillance, prevention, and mitigation strategies (WHO, 2023; WHO, 2022). OH seeks optimal health outcomes by recognising the interconnections and interdependencies between humans, animals, plants, and their shared environments (Prata et al., 2022). This integrated perspective emphasises that the health of one component directly affects the others, necessitating a holistic approach to address contemporary global health challenges.

OH provides a practical framework to tackle critical public health issues at local, regional, national, and global levels (Fasina et al., 2022). By mobilising expertise from multiple sectors, disciplines, and communities, OH enables collaboration to mitigate threats to health and ecosystems. The approach simultaneously addresses the collective need for clean water, air, and energy while advocating for safe and nutritious food, combating the adverse effects of climate change, and contributing to broader SDGs (WHO, 2022). These interconnected goals make OH essential in fostering resilience in health systems and environmental management, as illustrated in Figure 2.3 below.

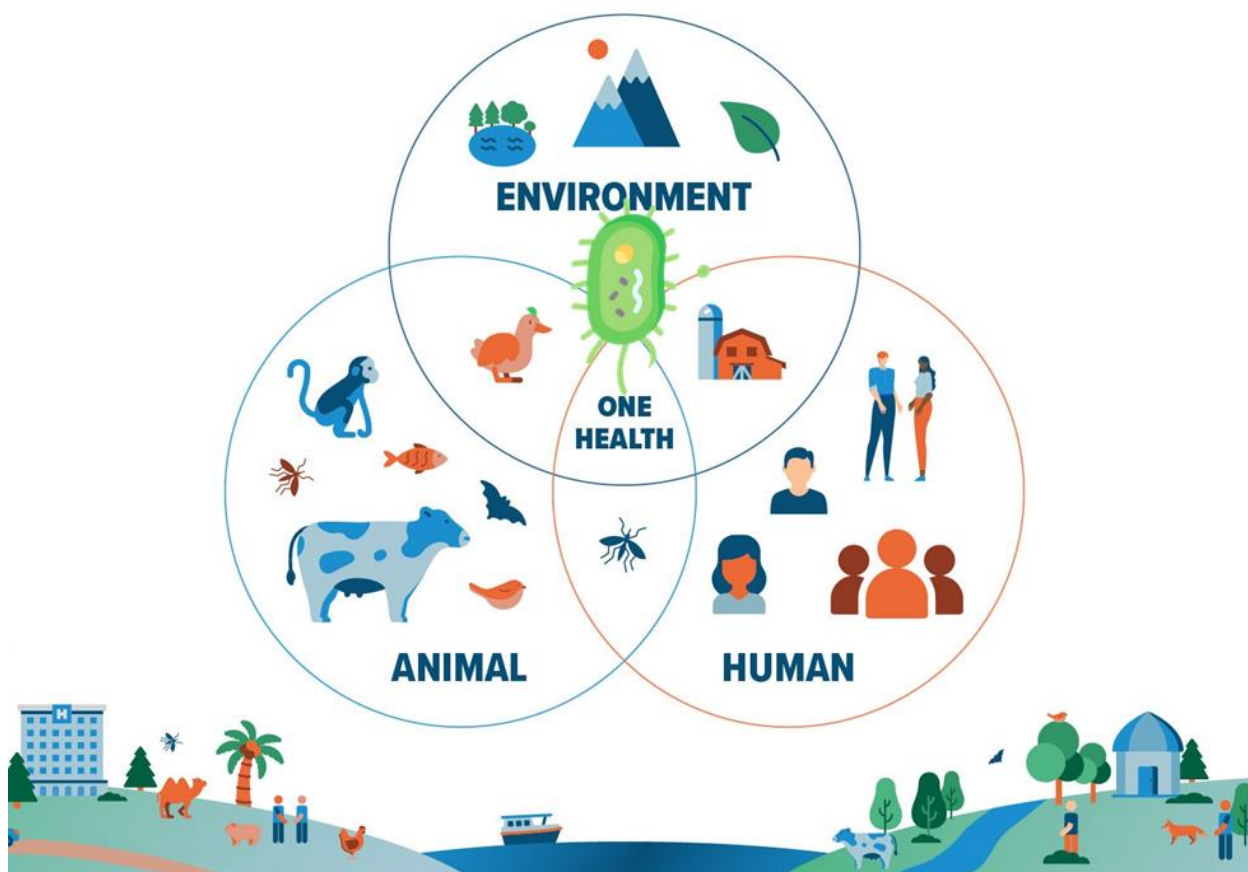


Figure 2.3: Microbial interaction between three sectors (human, animal and the environment) is interdependent according to the One Health framework.

Adapted from: (PREZODE, 2024) and https://www.flaticon.com/free-icon/bacteria_8986315, accessed on 8 December 2024.

A key strength of the OH framework is its ability to position health professionals as agents of change, empowering them to address determinants of health that extend beyond the scope of traditional medicine or public health alone (Rüegg et al., 2017). By promoting interdisciplinary collaboration, OH provides a platform to measure and manage complex determinants of health, integrating knowledge from fields such as veterinary medicine, environmental science, microbiology, epidemiology, and social sciences (Prata et al., 2022; Fasina et al., 2022). This approach ensures a synergistic response to public health challenges such as emerging infectious diseases, antimicrobial resistance, and environmental degradation, which are too complex to be effectively managed by any single discipline. By focusing on collaborative governance and shared responsibility, OH creates opportunities to improve health systems, enhance environmental conservation, and support economic and social resilience (WHO, 2022).

Table 2.1 The mechanism of action and target site for each of the 14 antibiotics selected for this study

Antibiotic class	Example of antibiotic	Target site	Resistance mechanism
B-lactams	Ampicillin	Targets penicillin binding proteins (PBPs). Inhibiting cell wall synthesis.	- Modification of PBP.
	Amoxicillin		- Efflux pumps.
	Cefazolin	Bactericidal.	- Membrane impermeability.
Aminoglycosides	Kanamycin	Binding of 30 ribosomal subunit.	- Modification of antibiotic by acylation, phosphorylation or adenylation.
	Neomycin	Disrupts translation.	
	Streptomycin	Inhibits protein synthesis.	
	Gentamycin	Bactericidal.	
Tetracycline	Tetracycline	Binding of 30 ribosomal subunit.	- Efflux pump.
		Inhibit protein synthesis.	- Ribosomal protection.
		Bacteriostatic.	- Enzymatic inactivation.
Fluoroquinolones	Ciprofloxacin	Targets the Gyr A subunit of DNA gyrase and topoisomerase IV.	- Efflux pumps.
	Nalidixic acid		- Target modification.
		Inhibition of DNA synthesis.	
		Bactericidal.	
Macrolides	Erythromycin	50S ribosomal subunit.	- Mutation in 23S rRNA.
		Inhibit protein synthesis.	- Efflux pumps.
		Bacteriostatic.	- Enzymatic inactivation.
			- Phosphotransferases.
			- Glycotransferases.
			- Esterases.
Trimethoprim	Trimethoprim	Dihydrofolate reductase.	- Resistant forms of the dihydrofolate reductase enzyme.
		Inhibit folate synthesis.	
		Bacteriostatic	
Sulphonamides	Sulphamethoxazole	Dihydropteroate synthase (DHPS).	- Resistant forms of DHPS enzymes.
		Inhibits folate synthesis.	- Mutations in <i>dhp</i> gene.
		Bacteriostatic	
Phenicol	Chloramphenicol	Inhibit protein synthesis.	- Acquisition of chloramphenicol acetyltransferase, preventing drug binding to target site.
		Inhibit peptide chain elongation.	

2.5.3 Multidrug resistance

In 2019, an estimated 4.95 million deaths worldwide were associated with ARB, with 1.27 million directly attributable to AR (Zhang et al., 2022; Murray et al., 2022). Among the various forms of AR, MDR is the most characterised phenomenon. MDR refers to bacterial resistance to treatment with multiple appropriate antimicrobial agents, defined as non-susceptibility to at least one drug in three or more antibiotic classes (see Table 2.1) (Turner et al., 2020; CDC, 2006). MDR results from bacteria either accumulating multiple resistance genes within a single cell or overexpressing multidrug efflux pumps (MDREPs), which actively expel a wide range of structurally and functionally unrelated antibiotics (Brown et al., 2019; Nikaido et al., 2009).

The implications of MDR are severe, with approximately 700,000 deaths attributed to MDR bacterial infections annually, a number projected to rise to 10 million by 2050 if resistance patterns continue to evolve unchecked (Serra-Burriel et al., 2020). This growing crisis carries significant economic consequences, with an estimated cost of 3.8% of the annual global GDP (approximately USD 1.2 trillion), potentially forcing 28.3 million people into extreme poverty (Serra-Burriel et al., 2020). Environmental monitoring has revealed that MDREPs are becoming increasingly prevalent within bacterial communities, further exacerbating the challenge of combating resistance (Brown et al., 2019).

Despite decades of intensive drug discovery efforts, few novel classes of antibiotics have been developed, mainly due to the stringent chemical, biological, and pharmacological requirements for effective antibiotic agents (Larsson et al., 2022; Wright et al., 2007). As such, therapeutic approaches are shifting towards combination therapies. These strategies involve combining or pairing antibiotics with adjuvants (such as clavulanic acid) that enhance drug efficacy by targeting resistance mechanisms. For example, β -lactamase inhibitors can neutralise bacterial enzymes that degrade β -lactam antibiotics, while adjuvants can interfere with bacterial signalling pathways such as two-component systems (Cacace et al., 2023; Adeniji et al., 2020; Tyers et al., 2019). Combination therapies provide an expanded selection of options for treating MDR infections, promising for overcoming drug resistance barriers (Medina et al., 2016; Worthington et al., 2013).

The rapid detection of AR and its quantification are prerequisites for controlling its spread and guiding effective treatment. Traditional phenotype-based methods, although time-consuming, remain the gold standard for AR detection (Frieri et al., 2017; Cooney et al., 2014). However, these methods are increasingly being complemented by molecular techniques such as high-throughput sequencing, polymerase chain reaction (PCR), gene microarrays, isothermal amplification, and mass spectrometry, which enable faster and more precise identification of resistance genes (Darby et al., 2023; Ding et al., 2024; Zhang et al., 2022; Koduru et al., 2019).

The advent of NGS has revolutionised medical microbiology, enabling comprehensive genotypic studies of resistance, allowing for the prediction and analysis of ARGs through tools like the Comprehensive Antibiotic Resistance Database (CARD) and the Resistance Gene Identifier (Koduru et al., 2019; Bertelli & Greub, 2013).

2.6 Methodological principles to isolate, characterise, and identify Enterobacteriaceae isolated from surface water

This section outlines the theoretical and practical framework used to isolate, characterise, and identify Enterobacteriaceae species originating from surface water bodies. The methodology has been subdivided into water quality monitoring, including physical and microbiological parameters, and phenotypical and genotypical methodologies, ensuring clarity and a systematic approach to the study.

2.6.1 Biosafety

Researchers have an ethical obligation to uphold good laboratory practices (GLP) and adhere to biosafety standards to ensure that their work does not exacerbate the problem being studied. Compliance with infection control protocols, containment measures, and safe waste disposal practices is essential to prevent further dissemination of pathogens, especially (MDR) bacteria, into the environment (Singer et al., 2016). Biosafety measures include the use of primary barriers (e.g., personal protective equipment, PPE) and secondary barriers (e.g., specialised biosafety work environments such as biosafety cabinets) (Burnett et al., 2009).

Historically, the lack of proper containment measures has led to significant biosafety incidents. For instance, the death of Dr Howard Taylor Ricketts in 1910 from typhus due to insufficient containment illustrates the critical need for effective biosafety protocols (CDC et al., 2004). Incidents involving exposure to hazardous microbes continue to occur, such as those reported in 2004 (Enserink & Kaiser, 2004). However, the biosafety field has advanced significantly, incorporating improved engineering controls, management policies, GLP training, preventive vaccinations, and immunisations, creating safer laboratory environments (Burnett et al., 2009).

2.6.2 Water Quality Monitoring

Water quality monitoring evaluates the state, suitability, and effectiveness of surface water by assessing physical and microbiological parameters. The water's suitability for various uses is determined by measuring the water against target water quality ranges provided by the DWS.

2.6.2.1 Physical parameters

2.6.2.1.1 Temperature

Temperature significantly influences the biological activity in water bodies. Rising temperatures are associated with an increase in microbiological indicator organisms, which is concerning given the effects of climate change (Delpla et al., 2016). Temperature levels are further influenced by geographical location, seasonal variations, and sampling depth (Makhlogh et al., 2008). Elevated temperatures affect microbial metabolic, nutritional, developmental, propagation, and distribution activities, highlighting the critical role of temperature in environmental microbiology (Elahi et al., 2015; Palamuleni & Akoth, 2015; Chandne, 2014).

2.6.2.1.2 pH

The pH of water, a logarithmic measurement of hydrogen ion concentration, determines whether water is acidic ($\text{pH} < 7$) or alkaline ($\text{pH} > 7$) (Yuqing & Keming, 2005). The optimal pH range for unpolluted surface water is between 6.5 and 8.5 (DWAF, 2004). Water outside this range can pose risks, such as pipe corrosion during transportation (WHO, 2007). High acidity in water bodies often results from acid mine drainage, particularly in regions near gold mines (Dallas & Day, 2004). pH also influences the solubility and biological availability of nutrients essential for microbial survival (Banks et al., 2004).

2.6.2.1.3 Total Dissolved Solids

Total dissolved solids (TDS) include inorganic salts and trace organic matter dissolved in water. Common sources include livestock runoff, sewage, and industrial wastewater (WHO, 2008; DWAF, 2004). Constituents such as bicarbonates, chlorides, nitrates, sodium, and magnesium are naturally derived from rocks, soils, and plant materials. Elevated TDS levels can indicate harmful contaminants like iron, manganese, sulphate, and arsenic, which are linked to health issues such as dizziness, irritability, and facial paralysis (Gupta et al., 2017; Dallas & Day, 2004).

2.6.2.2 Microbiological parameters

2.6.2.2.1 Total coliforms and *Escherichia coli* based on the membrane filtration technique (MFT)

Total coliforms, part of the Enterobacteriaceae family, are characterised by their ability to ferment lactose with gas production within 24 hours at 35 to 37°C. They are traditionally used as indicator organisms to assess water quality (WHO, 2011; Rompré et al., 2001). These organisms produce

β -galactosidase, resulting in yellow colonies on membrane lactose glucuronide (MLG) agar (WHO, 2008). While total coliforms are naturally present in water, soil, and sewage, their elevated levels in surface water indicate potential risk for respiratory and gastrointestinal illnesses (Haile et al., 1999). The membrane filtration technique (MFT) and MLG agar were also used to isolate *Escherichia coli* (*E. coli*). On this differential medium, *E. coli* colonies appear green due to the cleavage of 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (BCIG) by glucuronidase (Yates, 2020). As a recent faecal pollution indicator, *E. coli* highlights the contamination of water systems and poses the risk of urinary tract infections (UTIs), bacteraemia, and meningitis (WHO, 2011; Gabutti et al., 2000; Garizo-Hadzik et al., 2010; Paruch & Maehlum, 2012).

2.6.2.2.2 Total coliforms and *Escherichia coli* based on defined substrate technology (DST)

Defined substrate technology (DST), such as the Colilert-18® method, offers a less labour-intensive and faster alternative to MFT for detecting total coliforms and *E. coli* (Ramoutar, 2020). DST uses the substrate o-nitrophenyl- β -galactopyranoside (ONPG) for total coliforms and 4-methylumbelliferyl- β -D-glucuronide (MUG) for *E. coli*, as illustrated in Figure 2.4. Positive reactions result in yellow wells for coliforms and fluorescent wells for *E. coli* under UV light (Mobberley et al., 2022).

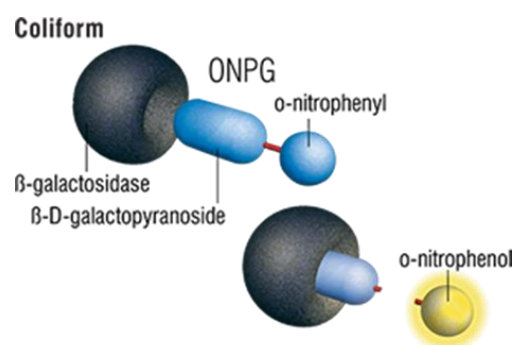


Figure 2.4: A visual presentation of the principle by which defined substrate technology functions to detect total coliforms and *E. coli* in water.

Accessed: <https://www.idexx.co.za/en-za/water/water-products-services/colilert/> on 1 April 2024.

2.6.3 Phenotypic methodology

This section focuses on the phenotypic methodologies applied in the selective and differential isolation of Enterobacteriaceae and the characterisation of their virulence factors. The methods include classical and advanced techniques to ensure accurate identification and profiling.

2.6.3.1 Selective and differential isolation

2.6.3.1.1 *E. coli* O157 isolation from water samples using the immunomagnetic separation technique (IMS)

The immunomagnetic separation (IMS) technique employs antibody-coated beads (Dynabeads) to isolate and concentrate *E. coli* O157 from water samples (Wright et al., 1994; Muller & Ehlers, 2005). Dynabeads anti-*E. coli* bind specifically to the target bacteria after incubation with an aliquot of the pre-enriched water sample. This method significantly reduces the interference of background flora, thereby lowering false-positive and false-negative results (Zhu et al., 2011). Following IMS, *E. coli* O157 colonies can be visualised on sorbitol MacConkey agar infused with antibiotics (Chapman et al., 1994; Bennett et al., 1995; Bolton et al., 1996; Heuvelink et al., 1997).

2.6.3.1.2 Sorbitol MacConkey agar with cefixime and potassium tellurite to culture pathogenic *E. coli*

Sorbitol MacConkey agar with cefixime and potassium tellurite (CT-SMAC) is a selective medium to isolate pathogenic and non-pathogenic *E. coli* (Muller, 2005). Cefixime inhibits the growth of *Proteus* spp., while potassium tellurite suppresses non-pathogenic *E. coli* (Zadik, 1993). Sorbitol replaces lactose as the fermentable sugar. Unlike non-pathogenic *E. coli*, the pathogenic serovar *E. coli* O157 does not ferment sorbitol, making it easily distinguishable on this medium (Manafi & Kremsmaier, 2001).

2.6.3.1.3 Rainbow agar O157 to culture pathogenic *E. coli*

Rainbow Agar O157 is a chromogenic medium with selective and differential properties, facilitating the rapid identification of pathogenic *E. coli* (Heuvelink, 2012). It contains substrates for two enzymes associated with *E. coli* O157: β -galactosidase and β -glucuronidase. Colonies expressing β -galactosidase appear blue-black, while those expressing β -glucuronidase are red (Bettelheim, 1998). The enzymatic activity is further confirmed using substrates such as p-nitrophenyl derivatives, enabling accurate differentiation (Jesudason, 2024).

2.6.3.1.4 Eosin methylene blue agar to confirm the presence of *E. coli*

Eosin methylene blue (EMB) agar is widely used to differentiate Enterobacteriaceae. It contains lactose and sucrose, which promote fermentation, leading to characteristic colony colour changes (Brenner et al., 1996; Arshad et al., 2006). For instance, *E. coli* produces a distinctive green metallic sheen, while other Enterobacteriaceae form purple or pink colonies (Lal & Cheeptham, 2007).

2.6.3.2 Extracellular enzyme production (virulence factors)

2.6.3.2.1 Sheep blood agar (5%) for pathogenic screening

Sheep blood agar detects haemolytic activity, an indicator of virulence in Enterobacteriaceae (Ahmed et al., 2010; Willey et al., 2011). This test is carried out on a medium composed of trypticase soy and 5% sheep blood, which promotes bacterial growth and produces the characteristic haemolytic patterns (Egwuatu et al., 2014; Yeh et al., 2009). Pathogenic species exhibit α -haemolysis (partial erythrocyte lysis) or β -haemolysis (complete erythrocyte lysis), which provides iron for bacterial growth (Mann, 2018; Willey et al., 2014).

2.6.3.2.2 Catalase

The catalase enzyme decomposes hydrogen peroxide into water and oxygen, protecting bacterial cells from oxidative damage (Willey et al., 2014). This activity indicates the bacteria's ability to survive oxidative stress, a key virulence factor (Huyche, 2002).

2.6.3.2.3 DNase

Pathogenic bacteria use degraded DNA as an energy source, a process facilitated by the presence of deoxyribonuclease (DNase), which is responsible for the hydrolysis of DNA into oligonucleotides (Palmer et al., 2012; Pavlov et al., 2004; Choi, 1987). Palmer (2012) highlighted that DNase expression in particular bacterial species confers resistance to host immune defences by degrading extracellular DNA, including chromatin filaments. This enzymatic activity impedes the formation and function of neutrophil extracellular traps (NETs), which are critical for immobilising and neutralising pathogens. Additionally, DNase is associated with a pathogen's resistance to phagocytic cells, such as macrophages and neutrophils, which engulf and destroy invading bacteria (Buchanan et al., 2005). By degrading DNA and evading host immune responses, DNase plays a significant role in the virulence and pathogenicity of these bacteria.

2.6.3.2.4 Gelatinase

Gelatinase is an extracellular zinc-endopeptidase encoded on an operon, which targets substrates such as insulin- β chains and haemoglobin (Su et al., 1999; Pillai et al., 2002). This enzyme plays a significant virulence role by hydrolysing structural proteins, including gelatine, collagen, casein, and other peptides, thereby facilitating tissue degradation and promoting bacterial invasion of the host's immune system (Park et al., 2008). By cleaving the fibrin layer surrounding bacterial cells, gelatinase further enables the dissemination of pathogens in host tissues (Waters et al., 2003). Additionally, Semendo (2003) observed that phenotypic expression

of the gelatinase gene is more prevalent in clinical isolates than environmental strains, indicating its critical role in pathogenicity and host colonisation.

2.6.3.2.5 Lecithinase

Lecithinase is an enzyme that hydrolyses lecithin, a group of fatty substances found in plant and animal tissues, including choline, fatty acids, glycerol, phosphoric acid, triglycerides, and phospholipids (Prinsloo, 2014). The enzymatic activity of lecithinase disrupts lecithin in plasma membranes, forming pores that facilitate the entry and spread of bacterial pathogens in host tissues (Willey et al., 2014). Esselman et al. (1961) characterised lecithinase activity by its ability to produce phosphorus and choline, leading to the precipitation of adipose tissue in bacterial cultures grown on agar. Furthermore, Sharaf et al. (2014) reported that lecithinase-producing bacteria may cause toxicity to reproductive tissues, erythrocytes, and cell membranes, highlighting their role in pathogenesis.

2.6.3.2.6 Lipase

Lipase is an extracellular enzyme capable of breaking down triacylglycerols into monoacylglycerols, diacylglycerols, free fatty acids (FFA), and glycerol (Bharathi et al., 2018; Knapp et al., 2016). This enzyme contributes significantly to bacterial pathogenicity by inducing host cell damage, modulating cellular processes, triggering inflammation, and interfering with cell signalling pathways (Bender & Flieger, 2010). Park et al. (2013) noted that lipase activity could target beneficial non-pathogenic bacteria, such as *Staphylococcus epidermis*, which play protective roles in the host. This disruption may allow foreign environmental pathogens to invade host cells, increasing the risk of infection.

2.6.3.2.7 Proteinase

Proteinases are responsible for initiating protein catabolism and, as a result, cause the breakdown of the peptide bonds of long protein chains that connect amino acids (Sharma et al., 2017). Culp and Wright (2017) stated that proteinase-producing bacteria lead to host infection and ultimately result in disease. This occurs because of diminished host cell defence systems, specifically the inactivation of immunoglobulin A (IgA), ultimately allowing passage of bacteria into host cells (Collin and Olsèn, 2003). The protein breakdown can be seen on agar by a visible clearance zone around the inoculum (Prinsloo, 2014).

2.6.3.2.8 Extended-spectrum β -lactamase (ESBL) chromogenic agar®

Extended-spectrum β -lactamases (ESBLs) are class A plasmid-mediated enzymes that hydrolyse a wide range of β -lactam antibiotics, including penicillins and third-generation cephalosporins (Boontham & Soontornrak, 2015). These antibiotics are essential for bacterial cell wall synthesis, particularly for constructing the peptidoglycan layer during environmental stress (Cho et al., 2014). ESBL-producing Enterobacteriaceae emerged in the 1980s and are considered among the most clinically significant antibiotic-resistant pathogens (Edwards, 2019). According to Coque et al. (2008), these bacteria are frequently associated with severe infections, including intra-abdominal sepsis, urinary tract infections, and hospital-acquired pneumonia. The detection of ESBL activity using chromogenic agar enables the identification of resistant bacterial strains, providing critical information for effective infection control and treatment strategies.

2.6.3.3 Antibiotic susceptibility profiling using the Kirby-Bauer disk diffusion assay

The Kirby-Bauer disk diffusion assay, developed in the 1950s, remains a cornerstone for evaluating bacterial antibiotic susceptibility (Hudzicki, 2009). Over the decades, the assay has undergone refinements to ensure its reliability, with the WHO establishing acceptable performance standards in 1961 (Gautam et al., 2013; Hudzicki, 2009). The Clinical and Laboratory Standards Institute (CLSI) has since taken the lead in recommending and standardising interpretive criteria, categorising bacteria as *susceptible*, *intermediate*, or *resistant* to specific antibiotics (Vineetha et al., 2015). These guidelines are pivotal for clinicians and researchers, providing a uniform framework for antimicrobial susceptibility testing (AST).

2.6.3.4 Serotyping assay using *E. coli* O157 latex kit

Serotyping is a key technique employed before evaluating virulence factors to identify pathogenic bacteria with high specificity (Lior, 1996). Among the most influential developments in serological classification, Kauffman's 1944 system remains a widely accepted framework for identifying and differentiating *Escherichia coli* strains. This classification system is based on three surface antigens: O (somatic), H (flagellar), and K (capsular), which collectively provide a serotype designation for bacterial strains. This study focused on the O and H antigens, explicitly targeting the O157:H7 serotype of *E. coli*. These surface antigens serve as markers for virulent clones, facilitating their identification and epidemiological tracking (Whittam et al., 1993).

2.6.3.5 EnteroPluri® assay

The EnteroPluri test is a commercial multi-sector biochemical identification system designed to characterise and identify bacterial species, particularly members of the Enterobacteriaceae

family. This test comprises a single, self-contained tube divided into 12 compartments, each containing a specific biochemical substrate. These compartments are used to assess the metabolic activities of bacterial isolates by testing their ability to perform various biochemical reactions. The test evaluates processes such as the fermentation of sugars (glucose, adonitol, lactose, arabinose, dulcitol, and sorbitol), decarboxylation of lysine and ornithine, reduction of sulphur, production of hydrogen sulphide, indole, and acetoin, deamination of phenylalanine, hydrolysis of urea, and utilisation of citrate. The test results are interpreted by observing colour changes in the compartments after incubation, which indicate positive or negative reactions for each biochemical test. These reactions generate a unique identification code, which is matched against the EnteroPluri-Test ©Codebook for the specific identification of the bacterial species. This system provides a rapid, cost-effective, and reliable means of identifying Enterobacteriaceae in clinical and environmental samples (Piccirilli et al., 2019).

2.6.3.5.1 Fermentation of sugars

The ability of bacteria to ferment carbohydrates is a fundamental metabolic property assessed by the EnteroPluri test. During fermentation, bacteria metabolise sugars such as glucose, adonitol, lactose, arabinose, dulcitol, and sorbitol to produce organic acids (e.g., acetic and lactic acids), alcohols, and gases (carbon dioxide and hydrogen). These metabolic byproducts result in a change in pH, which is visually indicated by a colour change in the test compartments. Acid production lowers the pH, causing the medium to change from red (alkaline) to yellow (acidic), signifying a positive fermentation result. For example, the yellow colouration observed with adonitol, lactose, arabinose, or sorbitol indicates the successful fermentation of these carbohydrates. Gas formation, a standard product of sugar fermentation, is also detected in the test system (Meng et al., 2022).

2.6.3.5.2 Urease

The urease test evaluates the ability of bacteria to hydrolyse urea into ammonia and carbon dioxide, utilising urea as a nitrogen source. This reaction is catalysed by the enzyme urease, which raises the pH of the medium, resulting in a colour change. Initially, the medium appears yellow, and urease production is indicated by a colour shift to red due to the alkaline pH. *Escherichia coli* and *Enterobacter* species typically do not produce urease, making this test an important discriminative tool for identifying species within the Enterobacteriaceae family (Barry and Badal, 1979).

2.6.3.5.3 Lysine and ornithine decarboxylation

This test determines the decarboxylation ability of bacteria, during which the enzyme decarboxylase removes the carboxyl group from lysine or ornithine, producing cadaverine and putrescine, respectively, along with carbon dioxide as byproducts. These alkaline byproducts raise the pH, which is indicated by a colour change from yellow to purple after incubation. These reactions are performed under anaerobic conditions, achieved by sealing the compartments with wax. This test is beneficial for distinguishing closely related bacterial species within the Enterobacteriaceae family (Li et al., 2021).

2.6.3.5.4 Citrate utilisation

This test detects those organisms that utilise citrate as a sole carbon source. These organisms produce alkaline metabolites, which are indicated by a visible colour change from green to deep blue (Bott, M., 1997). Enterobacteriaceae such as *Klebsiella* spp and *Salmonella* spp grow well on citrate-rich agar by the sodium-dependent pathway, resulting in acetate, formate and carbon dioxide (Shimamoto et al., 1991).

2.6.4 Genotypic methodology

The phenotypic approach to identifying Enterobacteriaceae, while widely used, is often limited due to the laborious nature of the process, the need for numerous tests, and its reduced sensitivity in distinguishing closely related species (Moore et al., 2006; Tang et al., 1998). To overcome these challenges, complementary genotypic methodologies have been developed, allowing for more precise identification of Enterobacterial species. These methods provide insight into the genetic makeup of organisms, including their virulence and AR profiles.

2.6.4.1 Identification of Enterobacteriaceae by 16S rRNA gene Sanger sequencing

The 16S ribosomal RNA (rRNA) gene, a highly conserved genetic marker, is universally present in Enterobacteriaceae, making it a reliable target for molecular identification (Janda and Abbott, 2007; Mignard and Flandrois, 2006). The amplification of the 16S rRNA gene via PCR is a widely used method due to its accuracy, feasibility, and simplicity (Joshi & Deshpande, 2010; Priest and Austin, 1993). According to Farnleitner (2001), the *uidA* housekeeping gene, encoding for β -D-glucuronidase, can also serve as a presumptive marker for Enterobacterial identification through PCR and gel electrophoresis.

PCR amplification generates multiple copies of target DNA, which can then be sequenced to determine the nucleic acid base sequence. This sequence is compared against the National

Centre for Biotechnology Information (NCBI) GenBank database using the Basic Local Alignment Search Tool (BLAST) for precise species-level identification (Burtscher et al., 2009). The 16S rRNA gene is particularly effective for distinguishing between closely related Enterobacterial genera due to its conserved and variable regions, which can be amplified using universal and species-specific primers (Hassan et al., 2014). Janda and Abbott (2007) further highlight its utility, noting its stability over time, universality, and sequence length of approximately 1,500 base pairs, which are essential for bioinformatics analyses and reliable identification.

2.6.4.2 Detection of virulence and antibiotic-resistant genes

The emergence of ARB has been a growing concern since Alexander Fleming's 1945 warning about the misuse of penicillin (Kohler et al., 1999). Research has confirmed that bacteria can acquire resistance through various mechanisms, including mobile genetic elements, spontaneous chromosomal mutations, and efflux pumps that decrease antibiotic concentrations in the cell (Portillo et al., 2000). The molecular detection of ARGs and virulence factors provides critical insight into the mechanisms underlying resistance and pathogenicity.

2.6.4.2.1 *Int1*

The class 1 integron gene (*Int1*) is a genetic marker indicative of ARGs (Koczura et al., 2016; Chen et al., 2015). Studies have linked the prevalence of *Int1* to anthropogenic pollution in both clinical and environmental settings (Gillings et al., 2014; Chen et al., 2013). Coertze and Bezuidenhout (2018) identified a co-occurrence of *Int1* with ARGs and metal resistance genes in surface water. The presence of *Int1* suggests resistance to aminoglycosides, β -lactams, and chloramphenicol, among other antibiotics (Lin et al., 2015). Alarming, conventional water treatment systems have proven ineffective in removing *Int1*, facilitating its persistence in surface water systems (Lin et al., 2015; Gillings et al., 2015).

2.6.4.2.2 *AmpC*

The *AmpC* gene, derived chromosomally in several Enterobacteriaceae species, encodes AmpC β -lactamase enzymes that confer resistance to cephalosporins (Ye et al., 2017). This resistance arises from either overexpression of the *AmpC* gene or the acquisition of mobile genetic elements containing the gene (Geyer et al., 2012; Perez-Perez and Hanson, 2002). This mechanism has been identified in *Citrobacter*, *Enterobacter*, *Escherichia coli*, and *Serratia* species, presenting significant challenges in antibiotic therapy (Farrokhnia et al., 2016; Shanthi et al., 2012).

2.6.4.2.3 *Sul1* and *Sul2*

The *sul* genes (*sul1* and *sul2*) are among the most widely distributed ARGs, conferring sulphonamide resistance (Muziasari et al., 2017; Miller et al., 2016; Colomer-Iluch et al., 2014). These genes encode dihydropteroate synthase enzymes that render sulphonamides ineffective (Chung et al., 2014). While *sul1* is commonly found on conjugative plasmids, *sul2* is associated with small, non-conjugative plasmids (Popowska and Krawczyk-Balska, 2013). The co-occurrence of *sul1* with *Int1* further amplifies its dissemination potential (Skold, 2000). Interestingly, *sul* genes have been regarded as naturally occurring in bacteria of raw water, calling for assessment of the prevalence in surface water (Calero-Caceres and Muniesa, 2016).

2.6.4.2.4 ESBL genes: *blaTEM* and *blaCTX*

Extended-spectrum β -lactamase (ESBL) genes such as *blaTEM* and *blaCTX* are concerning due to their ability to hydrolyse third-generation cephalosporins and β -lactams (Shahid et al., 2011). The gene *blaTEM* encodes allelic variants, while *blaCTX* originated from environmental bacteria and has been disseminated through plasmids (Bradford, 2001; Yan et al., 2001). These genes, often associated with transposons and integrons, have made β -lactams and cephalosporins ineffective as treatment options (Lachmayr et al., 2009; Sageerabanoo et al., 2015).

2.6.4.2.5 Carbapenemase genes: *blaOXA*, *blaKPC*, *blaNDM*

Carbapenemase genes, such as *blaOXA*, *blaKPC*, and *blaNDM*, confer resistance to carbapenems, a last-resort class of antibiotics (Gupta et al., 2011). According to Potter et al. (2016), resistance to these antibiotics can arise through changes in the membrane permeability by mutations in efflux pumps or porins coupled with ESBL expression or by expressing metallo- β -lactamases (*blaNDM*) or non-metallo-carbapenemases (*blaKPC* and *blaOXA*). The *blaOXA* gene encodes oxacillinases, while *blaKPC* and *blaNDM* produce enzymes that hydrolyse carbapenems, rendering them ineffective (Potter et al., 2016). These genes have been detected in *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Klebsiella pneumoniae*, presenting a global challenge in treating bacterial infections (Walsh, 2010).

2.6.4.2.6 The Shiga toxin virulent gene (*stx1* and *stx2*)

STEC is associated with the *stx1* and *stx2* virulence genes, commonly harboured by ruminants (Mughini-Gras et al., 2018). *Stx2* is more clinically severe and has a higher mortality rate (Yang et al., 2020). These genes are located on prophages integrated into the bacterial genome and are linked to gastrointestinal disorders, haemorrhagic colitis (HC), and haemolytic uremic syndrome (HUS) (Balasubramanian et al., 2019).

2.6.4.2.7 Intimin and haemolysin virulent gene (*eae* and *hlyA*)

Virulence genes such as *eae* and *hlyA* contribute to the pathogenicity of Enterobacteriaceae. The *eae* gene encodes intimin, a protein critical for bacterial attachment to enterocytes and the formation of attaching and effacing (A/E) lesions (Mora et al., 2005). The *hlyA* gene encodes haemolysin, enabling bacteria to lyse host erythrocytes and acquire iron for growth (Lorenz et al., 2013). These genes are strongly associated with the virulence profiles of pathogenic Enterobacteriaceae (Binandeh et al., 2020).

2.6.5 Whole genome sequencing of selected Enterobacteriaceae strains

The identification of Enterobacteriaceae isolated from water sources in this study has primarily relied on the conserved 16S rRNA gene. While this method is effective for general bacterial identification, it is limited in resolution, particularly when capturing genetic variation beyond this conserved region. Consequently, WGS is increasingly recognised as the gold standard for microbial identification, providing a more comprehensive and precise analysis at the species level (Salipante et al., 2015).

WGS entails sequencing the entire Enterobacterial organism's genome, delivering detailed insights into the genetic architecture, including coding and non-coding regions, repetitive elements, and structural variations (Gardner et al., 2015). This method facilitates a comparative genomic approach, enabling researchers to distinguish between closely related microbial strains with high precision (Karlsen et al., 2023; Besser et al., 2019). Such discriminatory power is invaluable for epidemiological investigations, where tracking the origin and spread of bacterial strains is crucial. Furthermore, WGS allows strain typing and addresses microbial diversity and evolution knowledge gaps.

Beyond species identification, WGS has emerged as a critical tool for characterising microbial strains regarding virulence and AR. For instance, WGS can identify genetic determinants associated with pathogenicity and resistance mechanisms, including those located on mobile genetic elements such as plasmids or transposons (Chenhaka et al., 2023; Heilbronner et al., 2011). This capability is critical in the context of MDR bacteria, as it supports the identification of resistance genes and their potential transfer mechanisms. Moreover, WGS has proven instrumental in identifying genetic markers for outbreak investigations and enhancing our understanding of bacterial adaptation to environmental and clinical pressures (Takeuchi et al., 2005; Zhang et al., 2003).

CHAPTER 3

MATERIALS AND METHODS

3.1 Description of study area

The study area encompasses two river catchments, the Mooi and Schoonspruit Rivers, and the Setumo Dam, all located in the North West province (NWP), South Africa. These aquatic ecosystems were chosen due to their strategic significance and susceptibility to anthropogenic influences, providing a robust foundation for evaluating water quality and the prevalence of Enterobacteriaceae species. A Garmin Nüvi 1310 global positioning system (Garmin, US) was employed to accurately geolocate the water sampling sites, as illustrated in Figures 6, 7, and 8. The red stars on the figures indicate the sites from which the 34 selected Enterobacteriaceae were isolated. The Mooi River watershed, depicted in Figure 3.2, derives its primary water sources from dolomitic eyes and springs. This catchment comprises three major tributaries: the Wonderfontein Spruit, the northern section of the Mooi River, and the Loop Spruit. The Mooi River basin consists of four significant dams, the Klerkskraal, Boskop, Klipdrift, and Potchefstroom dams (Molale, 2012). These surface water resources are essential for sustaining agricultural activities, urban development, and regional mining operations, including the economically vital gold and platinum mining sectors.

The Schoonspruit River, a tributary of the Vaal River, is fed by dolomite springs and spans a catchment area of 325 km². This river system is characterised by wetlands in its upper and lower reaches, while the Ventersdorp region forms a narrow channel (Molale, 2012; Fourie, 2017). The Schoonspruit River basin is home to the Klerksdorp Dam, another essential water body in the province, providing resources for local communities and agricultural irrigation. The Setumo Dam is part of the Molopo River system in Mahikeng. The Molopo River originates from the Molopo Eye, between Groot Marico and Lichtenburg. It initially flows westward, subsequently shifting southwest. The river's upper reaches fall within the Crocodile (West) and Marico WMAs, while its lower reaches form part of the Lower Vaal WMA. Key dams in the Molopo River system include the Disaneng, Modimola, and Setumo Dams (Akoth, 2018). The geographical context of the Setumo Dam is illustrated in Figure 3.3, which highlights the sampling sites under investigation. These surface water bodies support Mahikeng's urban development and local industries.

Sampling sites were selected to provide a representative water quality dataset across a gradient of anthropogenic activities. These activities include urban development, agricultural practices, and industrial operations, collectively affecting catchment water quality. The maps presented in this study were generated using ArcGIS Desktop 10.5 from the Environmental Systems Research

Institute (ESRI), ensuring high accuracy in spatial analysis (Johnston et al., 2001; Khan et al., 2018). Land-use data for the sampling sites were obtained from the South African National Land Cover Dataset for 2013–2014, provided by the Department of Environmental Affairs (DEA) (https://egis.environment.gov.za/sa_national_land_cover_datasets). Hydrological data, including dam and river characteristics, were sourced from the South African National Biodiversity Institute's (SANBI) National Freshwater Ecosystem Priority Areas (NFEPA) database (<ftp://ftp.csir.co.za/NRE/Ecosystems/NFEPA/>).

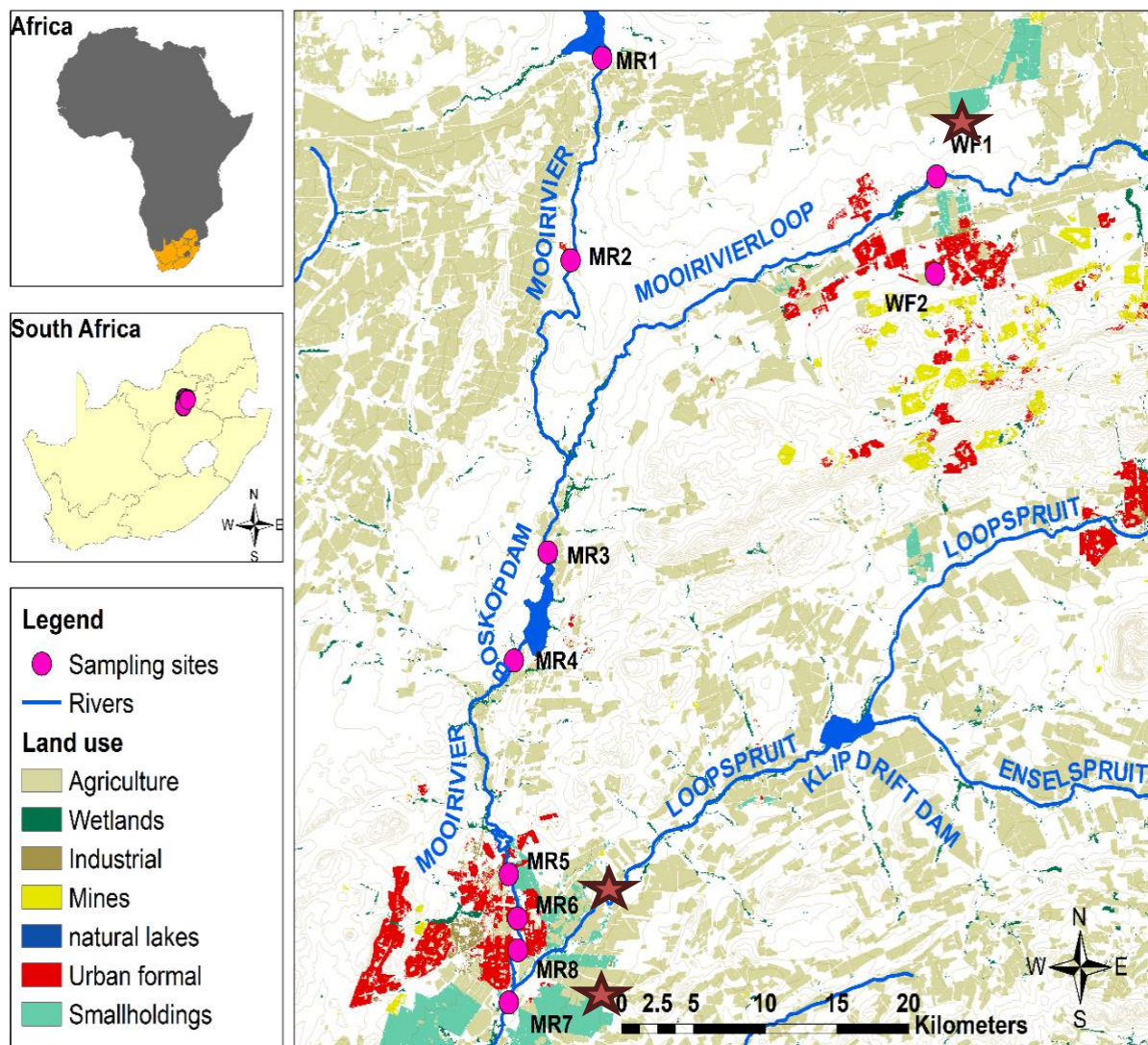


Figure 3.1: A detailed geographical map of the Mooi River and its surrounding area, illustrating the sampling sites in the North West province.

L. Bredenhann constructed the cartography and spatial analysis.

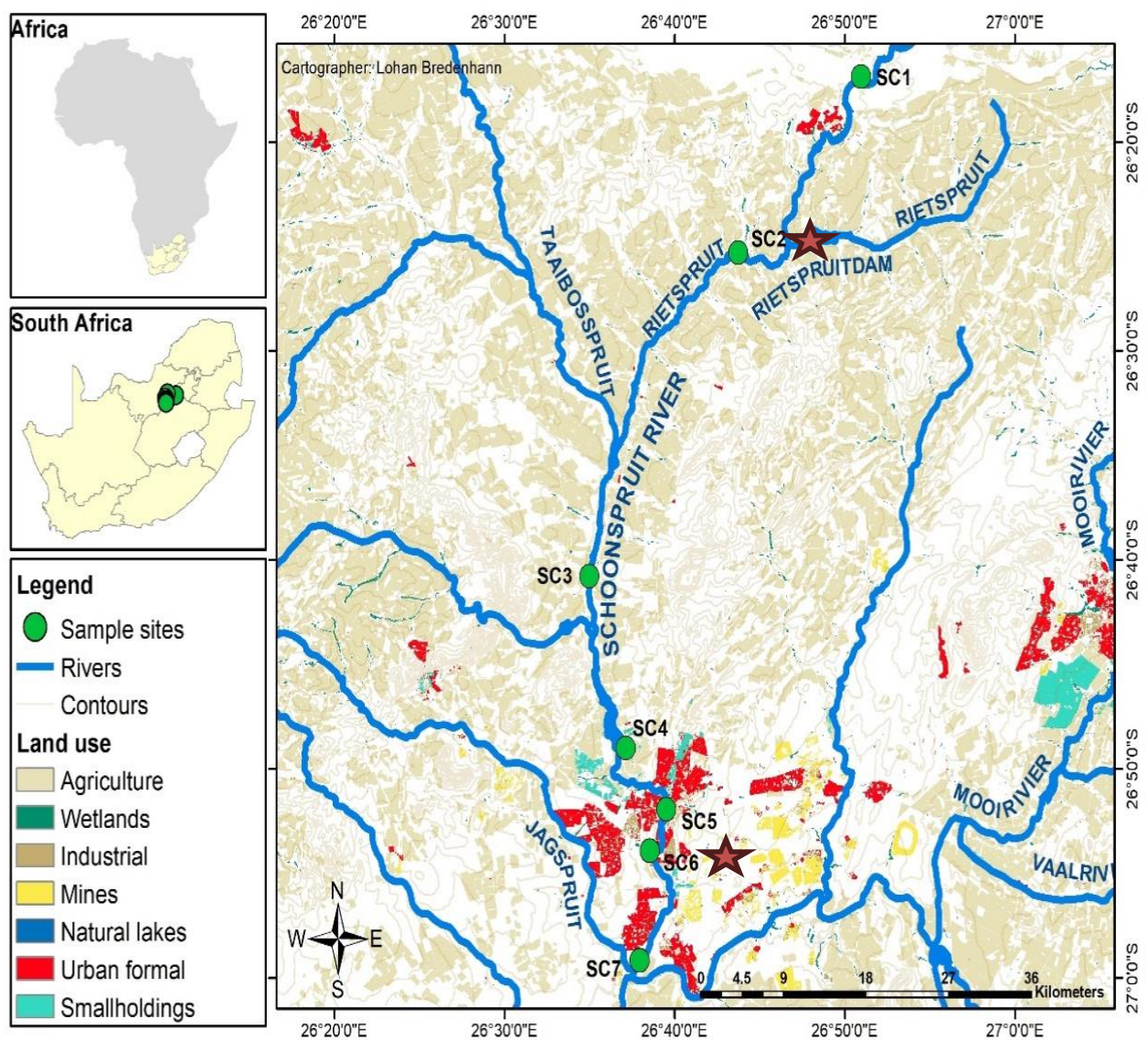


Figure 3.2: A detailed geographical map of Schoonspruit River and its surrounding area, illustrating the sampling sites in the North West province.

L. Bredenhann constructed the cartography and spatial analysis.

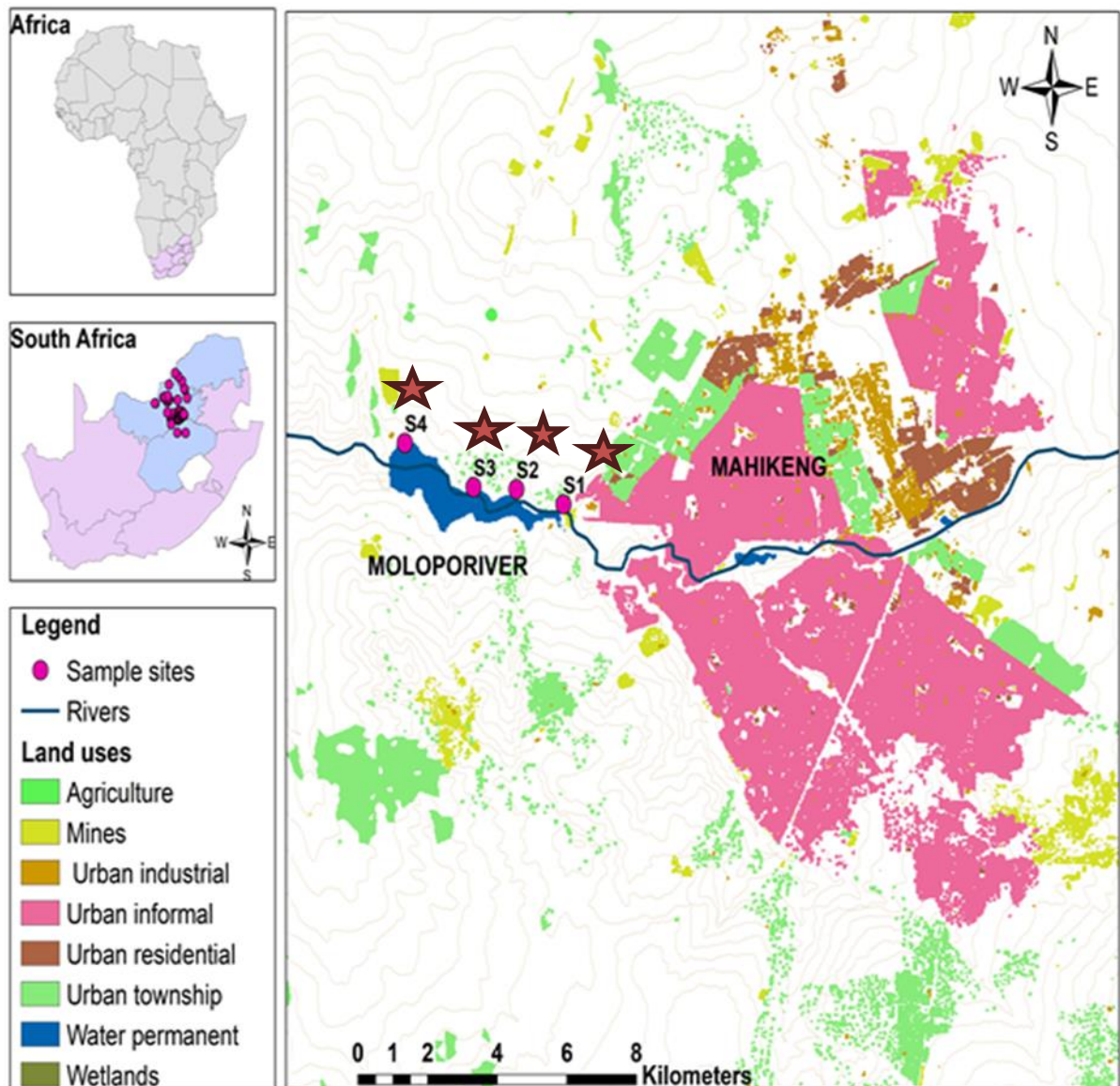


Figure 3.3: A detailed geographical map of Setumo Dam and its surrounding area, illustrating the sampling sites in the North West province.

L. Bredenhann constructed the cartography and spatial analysis.

3.2 Biosafety and occupational health considerations

Comprehensive biosafety and occupational health measures were implemented during this study to ensure the safety of personnel and the integrity of collected samples. The sampling process was meticulously planned, with researchers wearing appropriate personal protective equipment (PPE), including safety boots exceeding ankle height, nitrile gloves, a facemask, and a high-visibility bib (Figure 23). Additionally, 70% ethanol in a spray bottle was used as a surface and hand sanitiser to maintain aseptic conditions during sampling and processing (Burnett et al., 2009). Disposable PPE was disposed of in biohazard containers on return to the laboratory, and professional waste management services safely handled the disposal.

In the laboratory, biosafety level two (BSL-2) requirements were observed during all workflows, ensuring safe handling of potentially hazardous materials. The BSL-2 cabinet had a UV lamp for isolating and culturing organisms. This UV lamp was activated for 10 minutes before and after use to thoroughly decontaminate the cabinet (Homer et al., 2013). Decontamination was further reinforced by spraying and wiping the interior surfaces of the cabinet with 70% ethanol. The laboratory-grade paper used during the procedures was disposed of in biohazard containers.

Petri dishes used for culturing were also disposed of in biohazard containers and treated by the contracted disposal company. When glassware was used, the items were autoclaved to decontaminate cultures and then thoroughly cleaned and reused, reducing environmental impact while ensuring safety. These measures reflect the high biosafety standard and ecological responsibility integral to the research methodology.

3.3 Sample collection and physical parameter analysis

Surface water samples were collected during the wet season (October to May) in 2017, ensuring that sampling coincided with peak water flow periods. The sampling methodology was adapted to the physical characteristics of each site. Direct and dip sampling techniques were employed where sites were accessible, while tow or scoop-out methods, as outlined by the Environmental Protection Agency (EPA, 1994), were used when direct access was unavailable, such as on bridges.

Sterile (pre-autoclaved in the laboratory for an autoclave cycle of 20 minutes at 121°C) Schott bottles were used to collect water samples. Each bottle was labelled and immediately placed on ice in a portable cooler for transportation to the laboratory to preserve sample integrity (Valenzuela et al., 2020). At each sampling site, physical water quality parameters, including pH, temperature [°C], and total dissolved solids (TDS) [mg/L], were recorded on-site using the Oakton

PCS Testr™ 35 waterproof multi-parameter probe (Thermo Fisher Scientific, US). The probe was calibrated and operated according to the manufacturer's instructions to ensure accurate readings. Between measurements, the probe was rinsed with reverse osmosis-purified (RO) water and placed in a sterile glass beaker to avoid cross-contamination.

Sampling was conducted in triplicate in different sections of each site to account for spatial variability in water quality. Recorded data were documented in a field notebook and subsequently assessed against the Target Water Quality Ranges (TWQR) established by the Department of Water Affairs and Forestry (DWAF), now the DWS. TWQR benchmarks, summarised in the Annexure on Table A.1, were used as a reference to evaluate compliance with water quality standards.

3.4 Microbiological parameters

The methodologies used to isolate and identify Enterobacteriaceae in selected surface water bodies involved a combination of general and specific approaches. A selective approach targeted genera such as *Citrobacter*, *Escherichia*, *Serratia*, *Hafnia*, and other Enterobacteriaceae species, ensuring robust and precise microbiological characterisation.

3.4.1 Enumeration of Enterobacteriaceae using membrane filtration

Membrane filtration (MF) was used to isolate and enumerate Enterobacteriaceae from surface water samples using membrane lactose glucuronide agar (MLGA) (Oxoid, UK). Triplicate samples of 100 mL or their dilutions were filtered through 0.45 µm pore size, 47 mm diameter grid membranes (Pall Life Sciences, Mexico). The EZ membrane filtration unit (Millipore SAS, France) was decontaminated with 70% (v/v) ethanol, and funnels were rinsed with reverse osmosis (RO) water between different samples to avoid cross-contamination (Tula et al., 2022; Kritzinger, 2019).

Aseptic techniques were stringently followed when handling the mixed cellulose ester (MCE) membrane filters, and filtration was conducted in a fume hood. Following filtration, the membranes were placed on MLGA plates and incubated at 37°C for 24 hours. Colonies exhibiting green pigmentation on MLGA were identified and counted as *Escherichia coli* (*E. coli*). This selective methodology ensured reliable isolation and quantification of *E. coli* populations in the water samples. This method was used to process the water sampled from the Setumo Dam before procuring the Colilert system.

3.4.2 Enumeration of *E. coli* using defined substrate technology (DST)

The Colilert® 18 system (IDEXX, US) was used to detect and enumerate the most probable number (MPN) of Enterobacteriaceae, specifically targeting total coliforms and *E. coli*. Duplicate 100 mL water samples were mixed with Colilert-18 powder (IDEXX, US) in sterile bottles, forming a broth that was aseptically dispensed into Quanti-Tray/2000 (IDEXX, US) plates with 97 wells (49 large and 48 small). These trays were then incubated at 37°C for 18 to 24 hours.

After incubation, wells exhibiting yellow colouration, compared to a control, were counted to determine total coliform presence. *E. coli*-positive wells were further verified under UV light at a wavelength of 365 nm to detect fluorescence indicative of β -D-glucuronidase activity. The MPN per 100 mL sample was calculated using the MPN generator software available on the IDEXX platform (<https://www.idexx.com/en/water/resources/mpn-generator/>). This method offered rapid, efficient, and highly sensitive coliforms and *E. coli* detection in water samples. This technique was used for the two river systems since equipment and consumables were available during the October-November sampling period.

3.4.3 Enrichment, immuno-magnetic separation (IMS) and isolation of *E. coli* O157

A specific enrichment and separation protocol was followed to isolate *E. coli* O157. Water samples were enriched in modified buffered peptone water (mBPW). Briefly, 10 mL of sample was incubated at 37°C for 2 hours, followed by further incubation at 42°C for 20 to 24 hours without agitation. Adequate enrichment was indicated by turbidity in the solution, which was subsequently used for immuno-magnetic separation (IMS). The two river systems followed IMS and pre-enrichment methodology to supplement the DST. Dynabeads® anti-*E. coli* O157 (Invitrogen by Applied Biosystems, Norway) are superparamagnetic polystyrene beads coated with high-affinity anti-*E. coli* O157 antibodies via covalent bonding. A mixture of 20 μ L Dynabeads and 1 mL of enriched sample was incubated at room temperature (19 to 22°C) for 10 minutes in 2 mL microcentrifuge tubes.

The bead-bacteria complexes were recovered using a Chemagen magnetic stand (Chemagen Technologies by Perkin Elmer, Germany). The complexes were washed with a sterile wash buffer containing 0.05% (v/v) Tween®-20 (Merck, Germany) to remove non-specific material. The final 100 μ L bead-bacteria suspension was plated on Sorbitol MacConkey agar with cefixime and potassium tellurite (CT-SMAC) for presumptive *E. coli* O157 enumeration. IMS minimises interference from background flora, ensuring the selective isolation of motile and non-motile strains of *E. coli* O157 (Oporto et al., 2008). This technique is particularly valuable for achieving

high specificity and sensitivity in detecting and isolating pathogenic *E. coli* O157 from environmental samples.

3.4.4 Water quality index

Water quality was assessed by measuring physical parameters (temperature, pH, total dissolved solids [TDS]) and microbiological indicators (total coliforms and *Escherichia coli*) at sampled sites. The results were compared to Target Water Quality Ranges (TWQR) from the DWS (1996) for recreation, irrigation, and livestock watering (Table A.1). Due to the lack of TWQR for temperature and total coliforms, the assessment focused on pH, TDS, and *E. coli* levels. Sites exceeding TWQR thresholds were classified as having poor water quality, with the strictest limits applied to ensure compliance with health and environmental standards (APHA, 2017; WHO, 2011).

3.5 Phenotypic characterisation

3.5.1 Sorbitol MacConkey agar infused with cefixime and potassium tellurite (CT-SMAC)

Selected presumptive *Escherichia coli* (green) colonies from MLG agar plates were spot-inoculated onto CT-SMAC agar plates using aseptic techniques. The CT-SMAC agar plates were divided into sections to determine the percentages of putative *E. coli* O157 in a sample. Similarly, blue-fluorescent *E. coli* isolates from the Colilert-Quanti-Tray application were spot-inoculated onto CT-SMAC to estimate the prevalence of putative *E. coli* O157 at specific sampling sites. Pre-enriched samples (10 µL and 50 µL) that underwent IMS with Dynabeads anti-*E. coli* O157 were spread onto CT-SMAC plates, and the resulting colonies were enumerated to estimate the prevalence of putative *E. coli* O157.

Sorbitol MacConkey Agar (Oxoid, UK) was prepared according to the manufacturer's instructions and supplemented with 0.05 mg/L of cefixime trihydrate (Sigma Aldrich, India) and 2.5 mg/L of potassium tellurite (Sigma Aldrich, Japan). Plates were incubated at 37°C for 18 to 24 hours (EPA/600/R-10/056, 2010). Figure 26 shows that putative *E. coli* O157 colonies appear colourless due to an inability to ferment sorbitol, while non-pathogenic *E. coli* colonies appear pink (Ateba & Mbewe, 2014).

Putative *E. coli* O157 isolates (positive on CT-SMAC and showing α - or β -haemolysis) were sub-cultured onto tryptone soy agar (TSA) (Merck, Biolab) for purification. The isolates were streaked two to three times to ensure pure cultures and incubated at 37°C for 18 to 24 hours. Purified isolates were washed in 500 µL of Milli-Q water, serially diluted, and used for downstream characterisation. Morphological characteristics, such as shape, margin, elevation, size, appearance, and pigmentation, were observed on TSA. The isolates were temporarily stored at

4°C for further analysis. Following phenotypic and genotypic characterisation, these selected isolates were stored in a 25% glycerol stock solution for long-term storage at -80°C.

3.5.2 Rainbow agar O157

Rainbow agar O157 (Biolog, UK) is a selective and differential medium designed to isolate and rapidly identify enterohemorrhagic *Escherichia coli*. It differentiates serovars by producing distinct colony colours facilitated by chromogenic substrates specific for β -galactosidase and β -glucuronidase. The selectivity of the agar was enhanced by supplementing the medium with 0.8 mg/L of potassium tellurite and 10 mg/L of novobiocin (Sigma Aldrich, US). The medium was prepared by dissolving 30 g of dehydrated powder in 500 mL of reverse osmosis water, followed by sterilisation. Isolates were spot-inoculated, and colonies appearing black or grey after 24 hours of incubation at 37°C were confirmed as *E. coli* O157 (Verhaegen et al., 2015).

3.5.3 Confirmation of Enterobacteriaceae on EMB agar

Figure 25 shows eosin methylene blue (EMB) agar Levine (Oxoid, UK), which was used to confirm the identity of isolates as Enterobacteriaceae. The medium was prepared by dissolving 37.5 g of dehydrated powder in 1 L of reverse osmosis water, followed by sterilisation. Isolates were inoculated onto the agar and incubated for 24 hours at 37°C (Lal & Cheeptham, 2007). A green metallic sheen colony appearance indicated *E. coli*, whereas purple and pink would be other genera of the Enterobacteriaceae family (Oxoid, UK).

3.5.4 Serotyping of *E. coli* O157 isolates

Putative *E. coli* O157 isolates were tested against specific antisera to confirm agglutination reactions. A drop of 0.85% (w/v) saline solution (Merck, US) was placed on a reaction card and mixed with an overnight isolate culture. Blue latex particles sensitised with rabbit antibodies targeting the O157 somatic antigen were added. Isolates that formed clots were considered latex-positive (Renter et al., 2003). Non-pathogenic *E. coli* ATCC 10536 and pathogenic *E. coli* O157:H7 ATCC 700728 were used as negative and positive controls for all serological analyses (Figure 26 section k).

3.5.5 Extracellular enzyme assays

The extracellular enzyme activity of 34 selected Enterobacteriaceae isolates was assessed using solid media containing specific substrates. These media allowed for the detection of virulence-associated enzyme production (Tsholo, 2019; Horn, 2016; Molale, 2012).

3.5.5.1 Preliminary determination of pathogenicity: haemolysin cellular toxin

Putative *Escherichia coli* O157 isolates were spot inoculated onto 5% sheep blood agar plates (Thermo Scientific, RSA) and incubated at 37°C for 24 hours. The plates were examined for haemolytic activity, indicating the ability of isolates to lyse erythrocytes. α -haemolysis appeared as a greenish discolouration around the colony, while β -haemolysis presented as a clear zone (Beutin, 1991; Bielaszewska et al., 2014). Isolates viewed as putative *E. coli* O157 were spot inoculated onto 5% sheep blood agar plates, as visible in Figure 26 section J (Thermo Scientific, RSA). These plates were incubated for 24 hours at 37°C and investigated for their ability to lyse erythrocytes (Beutin, 1991; Bielaszewska et al., 2014).

3.5.5.2 Catalase activity

Catalase and peroxidase enzymes hydrolyse hydrogen peroxide (H_2O_2) into water (H_2O) and free oxygen (O_2). The catalase activity of pathogenic Enterobacteriaceae isolates was assessed on a sterile microscope slide and exposed to 3% (v/v) H_2O_2 . The release of gas bubbles indicated catalase-positive isolates (Reiner, 2010).

3.5.5.3 DNase activity

DNase agar (Oxoid, UK) was prepared according to the manufacturer's instructions. Plates were spot-inoculated with isolates and incubated at 37°C for 18 to 24 hours. After incubation, the plates were flooded with 1 M (Molar) HCl (Merck, USA) solution. The presence of a clear zone around the colonies indicated DNase activity.

3.5.5.4 Gelatinase activity

Gelatinase activity was assessed using agar plates containing 40 g/L Tryptone Soy Agar (Merck, Germany) and 3% (w/v) gelatin powder (Merck, Germany). Plates were spot-inoculated and incubated at 37°C for 24 to 48 hours. A clear or turbid halo surrounding the colonies indicated gelatinase activity.

3.5.5.5 Lecithinase activity

Lecithinase activity was tested on agar plates containing 40 g/L tryptone soy agar (Merck, Germany) supplemented with 100 mL of 50% egg yolk emulsion (Merck, Germany). Plates were incubated at 37°C for 24 to 48 hours. A distinct zone of cloudiness underneath or around the colonies suggested lecithinase activity.

3.5.5.6 Lipase activity

Lipase activity was determined using plates containing 40 g/L tryptone soy agar (Merck, Germany) supplemented with 1% (v/v) Tween-80 (Sigma Aldrich, Germany) after sterilisation. Plates were incubated at 37°C for 24 to 72 hours. A turbid halo around the colonies indicated lipase activity.

3.5.5.7 Proteinase activity

Proteinase activity was tested on agar plates containing 3% (w/v) skimmed milk (Oxoid, UK) mixed with Mueller-Hinton agar (35 g/L) (Merck, Germany). The skimmed milk and agar were sterilised separately and combined before being poured into plates. Spot-inoculated isolates were incubated at 37°C for 24 to 48 hours. A clear zone around the colonies indicated proteolytic enzyme activity.

3.5.5.8 Extended-spectrum β -lactamase-producing isolates

The detection of extended-spectrum β -lactamase-producing (ESBL) isolates was conducted using CHROMagar™ orientation medium, prepared by following the manufacturer's instructions. A concentration of 33 g/L of CHROMagar™ powder was diluted with reverse osmosis water and sterilised with an autoclave cycle of 15 minutes at 121°C. Once cooled to 50°C, 0.57 g/L of the ESBL supplement powder was added to enhance the selectivity of the medium. Isolates were spot-inoculated on the prepared ESBL plates using the lazy streak method (Figure 27). The plates were incubated at 37°C for 18 to 24 hours, and the resulting colony colours were used to interpret the identity of ESBL-producing bacteria. Pink colonies indicated *Escherichia coli* ESBL isolates, metallic blue colonies (with or without a red halo) suggested *Klebsiella*, *Enterobacter*, or *Citrobacter* ESBL, while brown colonies represented *Proteus* ESBL. Translucent cream-to-blue colonies indicated *Pseudomonas* ESBL and cream-to-opaque colonies represented *Acinetobacter* ESBL isolates (Girlich et al., 2019).

3.5.6 Disk diffusion of the 34 isolates to determine antibiotic susceptibility

The antibiotic susceptibility of 34 Enterobacterial isolates was determined using the Kirby-Bauer disc diffusion method (Bauer et al., 1966). A total of 14 antibiotics (Table 2.1) from eight classes were tested to screen for MDR. The antibiotics included β -lactams (ampicillin 10 μ g, amoxicillin 10 μ g, cefazolin 30 μ g), aminoglycosides (gentamycin 10 μ g, kanamycin 30 μ g, streptomycin 10 μ g, neomycin 30 μ g), tetracyclines (tetracycline 30 μ g), fluoroquinolones (ciprofloxacin 5 μ g, nalidixic acid 30 μ g), sulphonamides (sulphamethoxazole 100 μ g, trimethoprim 5 μ g), chloramphenicol (30 μ g), and macrolides (erythromycin 15 μ g).

An overnight bacterial culture was prepared in the nutrient broth and incubated at 37°C. Following incubation, a 100 µL inoculum of the bacterial culture was evenly spread onto Mueller-Hinton agar plates (Biolabs, USA). Antibiotic discs were aseptically placed on the agar surface, and the plates were incubated at 37°C for 18 to 24 hours. The results were assessed by measuring the inhibition zones around each antibiotic disc in millimetres (mm), which can be viewed in Table A.3. These measurements were interpreted using the Performance Standards for Antimicrobial Susceptibility Testing, M100 (2018), outlined by the Clinical and Laboratory Standards Institute (CLSI).

3.5.7 Biochemical analysis



Figure 3.4: This image shows the Enteropluri-test, a commercially available tool used to analyse the biochemical reactions of 34 different Enterobacterial strains.

The Enteropluri-Test (Liofilchem, Italy) was used to assess the biochemical properties of 34 Enterobacteriaceae strains. This commercially available standardised system facilitates the rapid identification of Enterobacteriaceae based on multiple biochemical reactions. The system comprises a 12-sector test tube (Figure 3.4) containing specialised culture media to assess 14 distinct biochemical reactions. These reactions include glucose fermentation, gas production, lysine decarboxylation, ornithine decarboxylation, adonitol fermentation, lactose fermentation, arabinose fermentation, sorbitol fermentation, dulcitol fermentation, urea hydrolysis, and citrate utilisation (Piccirilli et al., 2019).

The analysis was conducted following the manufacturer's instructions. Each of the 34 potentially pathogenic bacterial isolates was inoculated onto the Enteropluri-test sectors, and the test strips were incubated at 37°C for 18 to 24 hours. Following incubation, biochemical reactions were observed as colour changes in the individual compartments. Results were interpreted using the manufacturer-provided guidelines and scoring system, allowing for the precise identification of the isolates. The code generated was interpreted according to the manufacturer's instructions (<https://www.liofilchem.com/solutions/clinical/microbial-identification/id-systems/id-ast-systems-pure-colony/enteropluri-test>).

3.6 Genotypic characterisation

Molecular methods were employed to identify Enterobacteriaceae isolates, including genomic DNA extraction, amplifying the 16S rRNA and housekeeping genes, and subsequent phylogenetic analyses. These methodologies are detailed below.

3.6.1 Genomic DNA extraction, amplification and identification

Isolates confirmed as Enterobacteriaceae through culture methods were grown overnight in TSB (Merck, Germany) at 37°C. A 1 to 2 mL aliquot of the culture was centrifuged at 13,400 rpm, the supernatant was removed, and the resulting pellet was used for DNA extraction. The 16S rRNA gene was used for bacterial species identification.

3.6.1.1 Genomic DNA extraction

The Chemagic DNA extraction kit (Perkin Elmer, Germany) isolated total genomic DNA. This kit employs bead-magnetic separation to remove impurities, yielding a DNA-bead complex. The procedure followed the manufacturer's protocol. DNA quality and concentration (30–70 ng/μL) were measured using the NanoDrop™ One microvolume uv-vis spectrophotometer (Thermo Fisher Scientific, USA).

3.6.1.2 Amplification of the 16S rRNA gene and housekeeping gene (*uidA*)

The 16S rRNA and *uidA* genes were amplified using a PCR Max Alpha Cyclor 1 thermal cycler (Cole-Parmer, USA). The 25 μL reaction mix consisted of 2 μL template DNA (50 ng/μL), 1 μL 27F primer (10 pmol/μL) (Thermo Fisher Scientific, SA), 1 μL 1492R primer (10 pmol/μL) (Thermo Fisher Scientific, SA), 12.5 μL 2X DreamTaq Master Mix (Thermo Fisher Scientific, USA), and 8.5 μL DNase/RNase-free water (Thermo Fisher Scientific, USA). The thermal cycling conditions are summarised in Table 2.

3.6.1.3 Agarose gel electrophoresis

PCR products (3 μL) were visualised using 1.5% (w/v) agarose gel (SeaKEM® LE Lonza, USA) infused with 10 μL of 10 mg/mL ethidium bromide (EtBr) (Bio-Rad, UK). The gel was prepared in 50X TAE buffer (40 mM Tris, 20 mM acetic acid, and one mM EDTA; Merck, USA). Electrophoresis was conducted at 100 volts using a PowerPack (Bio-Rad, UK) for 30 minutes. The gel images were visualised with a Bio-Rad ChemiDoc imaging system (Hercules, USA).

3.6.1.4 Clean-up and PCR amplification of 16S rRNA gene amplicons

Amplified PCR products were purified using the AMPure XP PCR purification kit (Agencourt Bioscience Co., USA) following the manufacturer's protocol (Kieleczawa et al., 2006). The purified products were stored in sterile 0.2 µL PCR tubes for further analysis, and their quality was verified with the NanoDrop™ One microvolume uv-vis spectrophotometer system. In preparation for sequencing, a 20 µL PCR reaction included 1 µL DNA template (10–30 ng/µL), 4 µL ready-reaction (RR) mixture (1:10 dilution in nuclease-free water), 2 µL BigDye sequencing buffer, 1 µL 27F primer/1492R (3.2 pmol; Thermo Fisher Scientific, USA), and 12 µL nuclease-free water. The PCR-Max Alpha Cyclor conditions were as follows: 96°C for 60 seconds (initial denaturation), followed by 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 240 seconds for template elongation. This is known as sequencing PCR. Unlike traditional PCR, which amplifies a DNA region for analysis, sequencing PCR incorporates fluorescently labelled chain-terminating dideoxynucleotides (ddNTPs), found in the RR mixture, to generate fragments of varying lengths. Post-PCR products were purified using CleanSeq reaction bead solution (Agencourt Bioscience Co., USA) following the manufacturer's instructions (Wallis & Morrell, 2011).

3.6.1.5 Sanger sequencing, BLAST search and phylogenetic analysis

Purified products were loaded onto the SeqStudio Genetic Analyser (Applied Biosystems, USA). The resulting chromatograms were simultaneously analysed using Finch TV version 1.4 and BioEdit sequence alignment editor version 7.2.5. Sequences were compared to databases via the NCBI BLAST tool (<http://www.ncbi.nlm.nih.gov/BLAST>) and the EZTaxon database (<http://www.ezbiocloud.net>) for species identification. Selection criteria included e-values ≤0, percentage similarity ≥90%, and query cover ≥90%. Accession numbers for matches were recorded. Nucleotide sequences were edited for uncalled bases and reverse-complemented using BioEdit version 7.2.5 (Hall, 1999) and aligned using molecular evolutionary genetics analysis (MEGA) software version 11.0.10 (Tamura et al., 2021). A maximum parsimony tree was constructed, with bootstrap values derived from 100 replicates (Fourie, 2017; Molale, 2012). The maximum parsimony tree was the applicable choice for this study dataset, which included a moderate number of nucleotide sequences (Kumar and Nei, 2000; Felsenstein, 1985).

3.6.2 Detection of virulence genes from Shiga Toxin-producing *E. coli*

The DNA isolated from 34 Enterobacteriaceae strains was screened for key virulence genes associated with STEC. These genes included *stx1*, *stx2*, *eae*, and *hlyA*, as detailed in Table 3.1. PCR analysis was conducted using primers obtained from Inqaba Biotechnologies (South Africa),

and the screening was guided by established protocols (Morin et al., 2004; Reischl et al., 2002; Paton & Paton, 1998).

3.6.3 Antibiotic resistance gene screening and sequencing

3.6.3.1 Detection and Sanger sequencing of antibiotic resistance genes

The detection of ARGs, including those associated with extended-spectrum β -lactamase (ESBL) and carbapenem resistance, was conducted using conventional PCR. The PCR reaction mixture comprised 12.5 μ L of DreamTaq PCR master mix (Thermo Fisher Scientific, USA), 1 μ L of each primer (10 ng/ μ L) sourced from Inqaba Biotechnologies, 1 μ L of DNA template (20 ng/ μ L), and nuclease-free water, resulting in a final volume of 25 μ L. Thermal cycling conditions, primer sequences, and expected fragment lengths are detailed in Table 3.2 (Fadare & Okoh, 2021). The amplified products were loaded onto a 1.5% agarose gel containing a 10 mg/mL ethidium bromide solution and run at 80 V for 45 minutes. The electrophoresis gels were visualised using a Chemidoc imaging system (Bio-Rad, USA). This method facilitated the identification of resistance genes that contribute to AR in these isolates.

For further characterisation, the PCR products of the detected ARGs were subjected to Sanger sequencing at Inqaba Biotechnologies, RSA. The forward and reverse chromatograms obtained were processed and concatenated using BioEdit version 7.2.5, enabling full-length sequence assembly. The concatenated sequences were analysed through BLAST on the NCBI database to compare them with ARGs deposited in GenBank. This approach could confirm the identity of the resistance genes and provide a molecular basis for their functional role in AR.

3.7 Whole genome sequencing and computational methods

Comprehensive genomic insights were gained by subjecting four representative isolates from the predominant genus *Citrobacter* to WGS. DNA concentration and quality were measured using a Qubit 2.0 fluorometer (Thermo Fisher Scientific, USA), ensuring that high-quality DNA was available for downstream applications. Library preparation was performed using an Illumina DNA Preparation Kit, and sequencing was conducted on an Illumina MiSeq 2000 platform with a v3 flow cell (Makinde et al., 2022). The raw sequencing reads were subjected to quality filtering using Trimmomatic v0.36, followed by genome assembly with SPAdes v3.13.05. The quality of the assembled genomes and species designation was confirmed using Qualimap (<http://qualimap.bioinfo.cipf.es/>), while genome annotation was performed using Prokka (<https://github.com/tseemann/prokka>).

The identification of resistance and virulence genes was conducted using specialised bioinformatics tools. ResFinder v4.1 and the CARD were employed to identify ARGs, while VirulenceFinder v2.0 (Centre for Genomic Epidemiology) was used to detect virulence factors. Multi-locus sequence typing was performed using PubMLST (<https://github.com/tseemann/mlst>), and functional annotations were confirmed using the RAST platform (Rapid Annotation using Subsystem Technology, <https://rast.nmpdr.org/>). Additionally, KEGG pathway analysis was integrated with phenotypic data to elucidate the functional relevance of identified genes.

3.8 Statistical analysis

Data generated in this study were recorded and organised using Microsoft Excel (Microsoft Corporation, USA). Descriptive statistics, including means and standard deviations, were calculated for triplicate samples to summarise the data (Field, 2018). Advanced statistical analysis was conducted using IBM SPSS Statistics (Version 29.0.2.0, IBM Corporation, 2023, Armonk, NY). Pearson's correlation coefficient was employed to evaluate the strength and direction of the linear relationship between the recovery rates of *Escherichia coli* O157 from *E. coli* isolates. The significance of the correlation was assessed by calculating the p-value, with a threshold of $p < 0.05$ determining the statistical significance (Frost, 2021).

A one-way analysis of variance (ANOVA) test was done to compare the variability in the recovery of *E. coli* O157 between the two methods (membrane filtration and defined substrate). The ANOVA determined whether statistically significant differences existed between the means of these two groups (Bland and Altman, 1995). An F-statistic value was applied to identify specific group differences (Frost, 2021; Zar, 2010; Tukey, 1949)— parametric correlation analysis and variance testing provided a comprehensive data analysis. Pearson's correlation assessed the relationship between recovery rates, while ANOVA facilitated the evaluation of method-specific differences. These statistical methods ensured robust and reliable data interpretation, adding validity to the study's conclusions.

Table 3.1: Universal 16S rRNA gene, Enterobacterial housekeeping, virulence oligo primer sequence, and PCR conditions

Primer	Sequence (5'-3')	Specificity	Amplicon size (bp)	PCR cycling conditions
<i>27F</i>	AGAGTTTGATCMTGGCTCAG	16S bacterial ribosomal genes	1465 ^a	95°C for 300 seconds
<i>1492R</i>	CGGTTACCTTGTTACGACTT			95°C for 30 seconds 52°C for 30 seconds 72°C for 60 seconds 72°C for 180 seconds
<i>uidA F</i>	CCGATCACCTGTGTCAATGT	Enzyme β -D-glucuronidase	380 ^b	95°C for 300 seconds
<i>uidA R</i>	GTTACCGCCAACGCGCAATA			95°C for 30 seconds 60°C for 60 seconds 72°C for 120 seconds 72°C for 600 seconds
<i>eae F</i>	GACCCGGCACAAGCATAAGC	Intimin Gene	384 ^e	95°C for 600 seconds
<i>eae R</i>	CCACCTGCAGCAACAAGAGG			94°C for 60 seconds 59°C for 60 seconds 72°C for 60 seconds 72°C for 600 seconds
<i>hlyA F</i>	GCATCATCAAGCGTACGTTCC	Haemolysin plasmid-encoded Gene	534 ^e	95°C for 600 seconds
<i>hlyA R</i>	AATGAGCCAAGCTGGTTAAGCT			94°C for 60 seconds 59°C for 60 seconds 72°C for 60 seconds 72°C for 600 seconds
<i>stx1 F</i>	ATAAATCGCCATTCGTTGACTAC	Shiga Toxin Gene subunit	180 ^e	95°C for 600 seconds
<i>stx1 R</i>	AGAACGCCCACTGAGATCATC			94°C for 60 seconds 63°C for 60 seconds 72°C for 60 seconds 72°C for 600 seconds
<i>stx2 F</i>	GGCACTGTCTGAACTGCTCC	Shiga Toxin Gene subunit	255 ^e	95°C for 600 seconds
<i>stx2 R</i>	TCGCCAGTTATCTGACATTCT			94°C for 60 seconds 63°C for 60 seconds 72°C for 60 seconds 72°C for 600 seconds

See Muyzer et al. (1993) or Weisburg et al. (1991^a); Martins et al. (1993^b); and Paton and Paton (1998^e).

Table 3.2: Antibiotic-resistant target site oligo primer sequence and PCR conditions

Primer	Sequence (5'-3')	Specificity	Amplicon size (bp)	PCR cycling conditions
<i>Int1 F</i>	CTGGATTTTCGATCACGGCACG	Class integron- integrase gene	1 473 ^w	95°C for 300 seconds
<i>Int1 R</i>	ACATGCGTGTAATCATCGTCG			94°C for 30 seconds 49°C for 30 seconds 72°C for 60 seconds 72°C for 420 seconds } 35x
<i>AmpC F</i>	TTCTATCAAMACTGGCARCC	Confer Ampicillin Resistance	550 ^v	94°C for 300 seconds
<i>AmpC R</i>	CCYTTTTATGTACCCAYGA			94°C for 30 seconds 49°C for 30 seconds 72°C for 60 seconds 72°C for 420 seconds } 33x
<i>Sul1 F</i>	CGTGGGCTACCTGAACGATA	Confer Sulphamethox azole Resistance	721 ^w	95°C for 240 seconds
<i>Sul1 R</i>	AAGAACCGCACAATCTCGTC			95°C for 60 seconds 66°C for 60 seconds 72°C for 60 seconds 72°C for 420 seconds } 35x
<i>sul2 F</i>	GATATTCGCFFTTTTCCAGA	Confer Sulphamethox azole Resistance	721 ^w	95°C for 240 seconds
<i>sul2 R</i>	CAAAGAACGCCGCAATGT			95°C for 60 seconds 66°C for 60 seconds 72°C for 60 seconds 72°C for 420 seconds } 35x
<i>blaTEM F</i>	TCGCCGCATACACTATTCTCAGAAT GA	ESBL Gene	445 ^x	95°C for 900 seconds
<i>blaTEM R</i>	ACGCTCACCGGCTCCAGATTTAT			94°C for 30 seconds 50°C for 30 seconds 72°C for 120 seconds 72°C for 600 seconds } 30x
<i>blaCTX F</i>	ATGTGCAGYACCAGTAARGTKATG GC	ESBL Gene	593 ^x	95°C for 900 seconds
<i>blaCTX R</i>	TGGGTRAARTARGTSACCAGAAAYC AGCGG			94°C for 30 seconds 58°C for 30 seconds 72°C for 120 seconds 72°C for 600 seconds } 30x
<i>blaOXA F</i>	GCGTGGTTAAGGAATGAACAC	Carbapenema se Gene	438 ^y	94°C for 600 seconds
<i>blaOXA R</i>	CATCAAGTTCAACCCAACCG			94°C for 30 seconds 52°C for 40 seconds 72°C for 50 seconds 72°C for 300 seconds } 36x
<i>blaKPC F</i>	CGTCTAGTTCTGCTGTCTTG	Carbapenema se Gene	798 ^y 232 ^y	94°C for 600 seconds
<i>blaKPC R</i>	CTTGTCATCCTTGTTAGGCG			94°C for 30 seconds 52°C for 40 seconds 72°C for 50 seconds 72°C for 300 seconds } 36x
<i>blaNDM F</i>	GGTTTGGCGATCTGGTTTTTC	Carbapenema se Gene	621 ^y	94°C for 600 seconds
<i>blaNDM R</i>	CGGAATGGCTCATCACGATC			94°C for 30 seconds 52°C for 40 seconds 72°C for 50 seconds 72°C for 300 seconds } 36x

See Coertze and Bezuidenhout (2018); Labbate et al. (2008^v); Lanz et al. (2003^w); Monstein et al. (2007^x); and Poirer et al. (2011^y)

CHAPTER 4

RESULTS AND INTERPRETATION

4.1 Introduction

The water quality of selected surface water systems, namely the Setumo Dam, Mooi River, and Schoonspruit River, was evaluated using Enterobacteriaceae coliforms and in situ physical parameter measurements. A total of 20 sampling sites were monitored during the warm and rainy season of 2017. This study aimed to characterise the potential pathogenic Enterobacteriaceae distributed in these water bodies that could pose health risks to humans and animals through direct or indirect contact. The results of this investigation are organised into three key sections: water quality; phenotypic characteristics, genotypic identification and characteristics; and the comprehensive characteristics of the predominant genera revealed through WGS.

4.2 Water quality

4.2.1.1 Physical parameters



Figure 4.1: Line chart illustrating pH levels measured at each sampling site for the three surface water systems

Definition: 1: WF1, SCH1, S1; 2: WF2, SCH2, S2; 3: MR1, SCH4, S3; 4: MR2, SCH5, S4; 5: MR3, SCH6; 6: MR4, SCH7; 7: MR5; 8: MR6; 9: MR7; 10: MR8

Figure 4.1 presents a line chart illustrating the pH levels measured across the 20 sampling sites in the Setumo Dam, Mooi River, and Schoonspruit River systems. The pH values ranged from 7.67 to 9.24, with the highest value recorded at Site SCH4, a recreational area along the Schoonspruit River. This value exceeded the recommended acceptable range (Table A.1) for pH (6.5–8.5) specified by the DWS (1996). The second-highest pH value was observed at Site S4 in the Setumo Dam, located near a drinking water treatment works. On average, the Mooi River recorded the lowest pH (8.23 ± 0.25). At the same time, the Setumo Dam exhibited the highest average pH (8.55 ± 0.63), as shown in Table A.2. Notably, the Mooi River was the only system where pH levels consistently fell within the acceptable range, indicating better compliance with water quality standards.

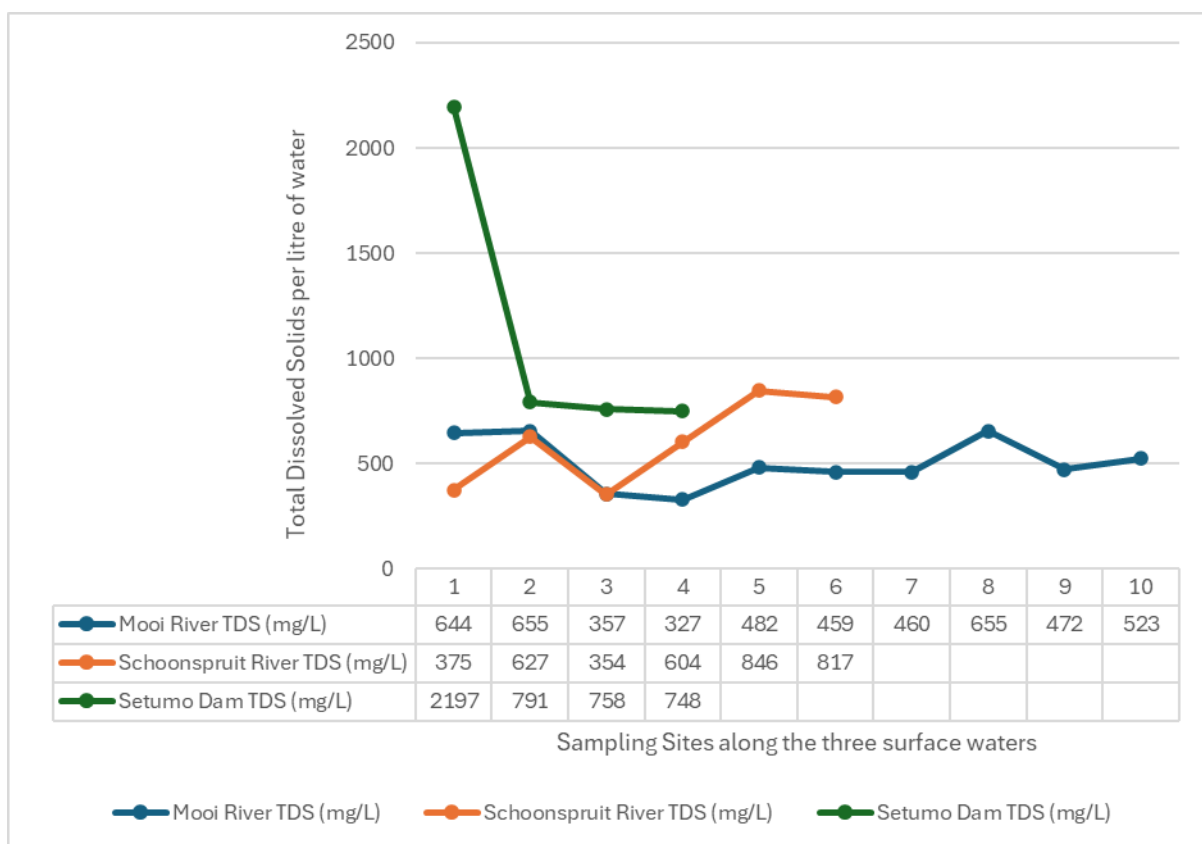


Figure 4.2: Line chart of TDS levels measured at each sampling site for the three surface water systems

Definition: 1: WF1, SCH1, S1; 2: WF2, SCH2, S2; 3: MR1, SCH4, S3; 4: MR2, SCH5, S4; 5: MR3, SCH6; 6: MR4, SCH7; 7: MR5; 8: MR6; 9: MR7; 10: MR8

Figure 4.2 illustrates the total dissolved solids (TDS) levels across the sampling sites. TDS values ranged from 327.00 mg/L to 2197.00 mg/L, with the highest concentration recorded at Site S1 of

the Setumo Dam. This site, situated downstream of a WWTP, exceeded the target water quality range (TWQR) for agricultural activities, including irrigation and livestock watering (refer to Table A.1 in the annexure). The Mooi River exhibited the lowest average TDS (503.40 ± 117.36 mg/L), while the Setumo Dam recorded the highest (1123.50 ± 715.90 mg/L), as summarised in Table A.2. TDS levels in the Mooi and Schoonspruit Rivers were generally suitable for livestock watering. Still, the elevated TDS levels in the Setumo Dam exceeded the TWQR, highlighting concerns about its suitability for agricultural use.

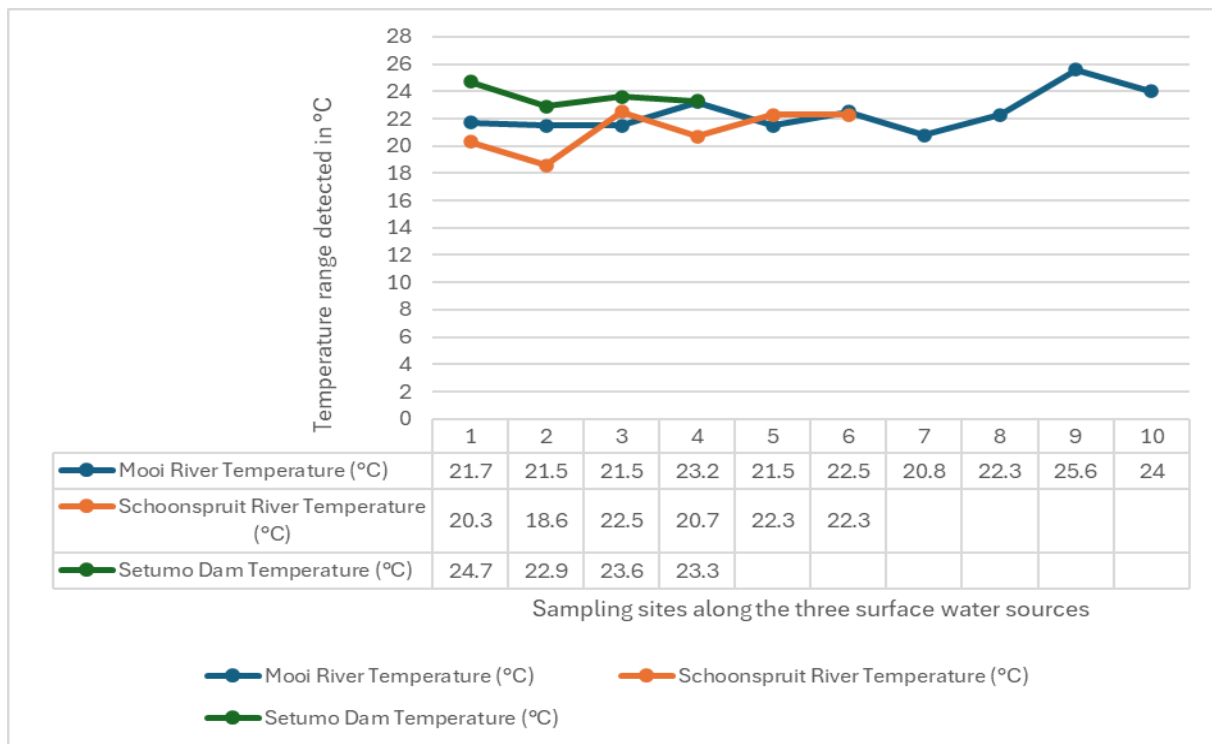


Figure 4.3: Line chart illustrating the temperature levels measured at each sampling site for the three surface water systems

Definition: 1: WF1, SCH1, S1; 2: WF2, SCH2, S2; 3: MR1, SCH4, S3; 4: MR2, SCH5, S4; 5: MR3, SCH6; 6: MR4, SCH7; 7: MR5; 8: MR6; 9: MR7; 10: MR8

Figure 4.3 shows the temperature levels of the three surface water systems, which ranged from 18.60°C to 25.60°C . The lowest temperature was recorded at the Bodenstein site (SCH2) along the Schoonspruit River, while the highest was observed at Site MR7 along the Mooi River. Average temperatures were lowest in the Schoonspruit River ($21.12 \pm 1.54^{\circ}\text{C}$), followed by the Mooi River ($22.46 \pm 1.45^{\circ}\text{C}$), and highest in the Setumo Dam ($23.63 \pm 0.77^{\circ}\text{C}$). While temperature data were not directly compared to TWQRs due to the lack of explicit guidelines for recreational and agricultural uses, the recorded values reflect seasonal and environmental influences.

4.2.1.2 Microbiological parameters

4.2.1.2.1 Defined substrate technology

Defined substrate technology was employed to quantify total coliforms and *Escherichia coli* in water samples collected from the Mooi and Schoonspruit Rivers. The most probable number (MPN) of total coliforms in a 100 mL sample was lowest at the Klerkskraal site (MR2) along the Mooi River. High levels of total coliforms were detected all along the Mooi River, with an average of 2332.95 MPN/100 mL. In the Schoonspruit River, the lowest total coliform count was recorded at the Ventersdorp raw water site (SCH1), which serves as the river's spring and origin. Another site with reduced MPN of total coliforms was the Voortrekker Road site (SCH5), located in a less densely populated area. The average total coliform count along the Schoonspruit River was 1929.38 MPN/100 mL, slightly lower than the Mooi River.

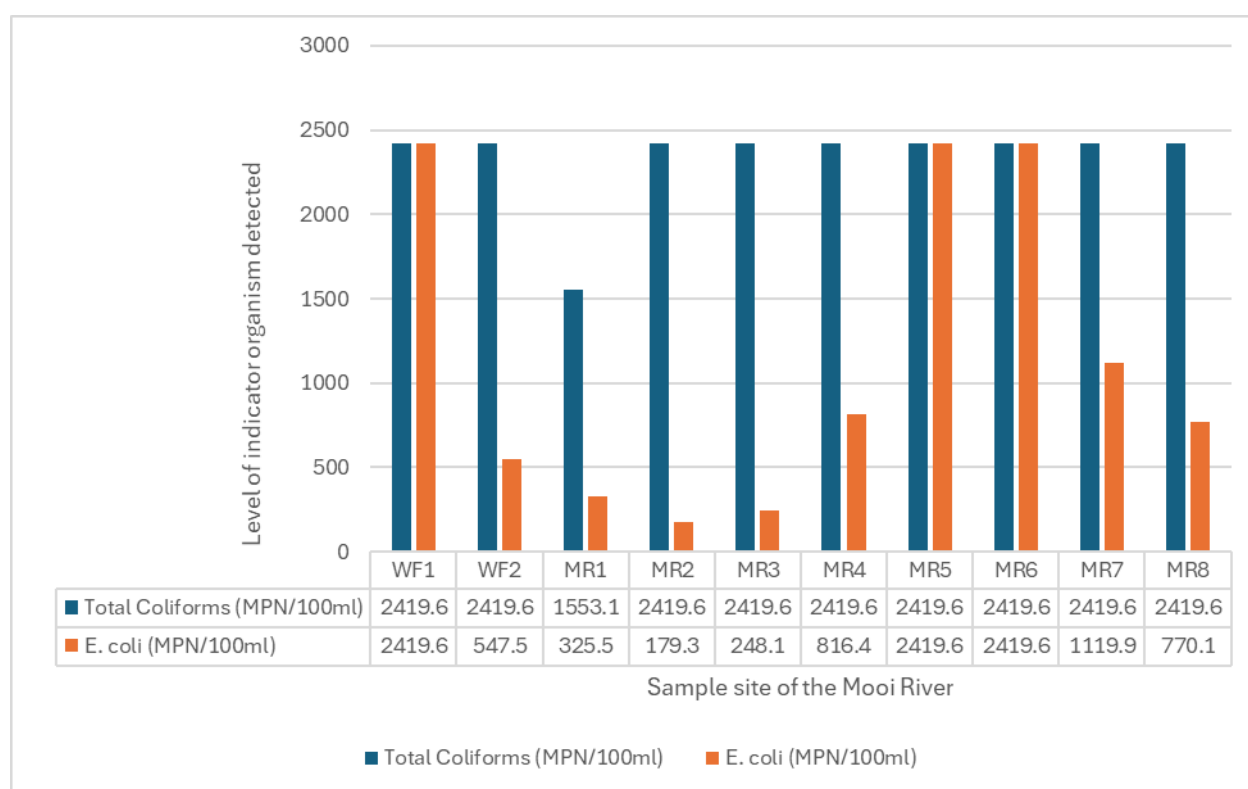


Figure 4.4: Bar chart of microbiological parameters measured across the 10 sites located along the Mooi River

Figure 4.4 presents the microbiological parameters measured across the 10 sites along the Mooi River. *E. coli* levels, which indicate faecal contamination, were also assessed. While the two Wonderfontein spruit sites (WF1 and WF2) showed no differences in total coliforms, a marked

reduction in *E. coli* levels was observed between these sites, dropping from 2419.60 to 547.50 MPN/100 mL. The lowest *E. coli* count in the Mooi River was detected at the Klerkskraal site (MR1), with 179.30 MPN/100 mL, followed by a slight increase at the Muiskraal site (MR2). Elevated *E. coli* levels were observed at the Thabo Mbeki Bridge (MR5) and Trimpark Bridge (MR6), influenced by urban activity and anthropogenic pollution. A gradual reduction in *E. coli* levels was noted downstream, potentially due to dilution or natural attenuation.

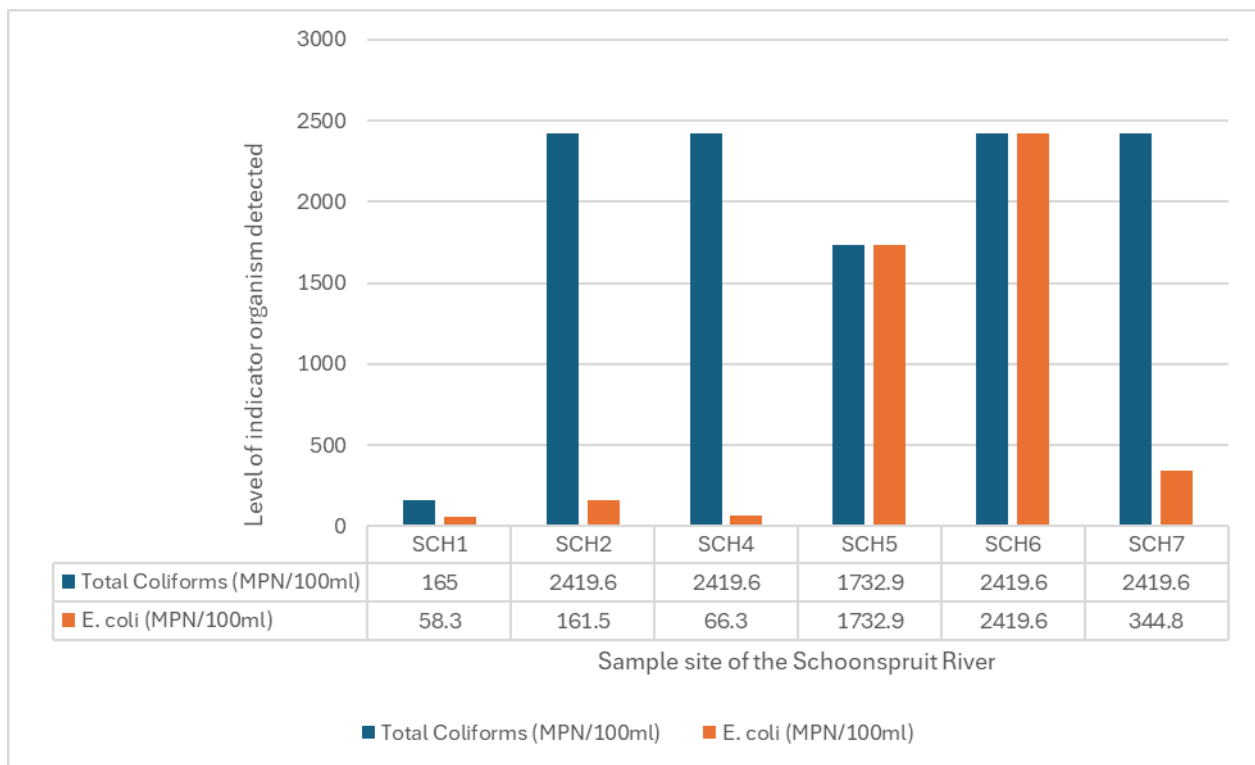


Figure 4.5: Bar chart of microbiological parameters measured across the seven sites along the Schoonspruit River.

Site 3 had no water when sampling, and site 4 is the Klerksdorp dam.

Figure 4.5 highlights the microbiological parameters along the Schoonspruit River. Overall, *E. coli* levels in this river were below 344.80 MPN/100 mL at most sites. However, the Voortrekker Road site (1732.90 MPN/100 mL) and the site downstream of the WWTP (2419.60 MPN/100 mL) exhibited elevated *E. coli* counts. These locations, characterised by urban activity and wastewater discharge, demonstrate the impact of anthropogenic influences. Although the average *E. coli* count in the Schoonspruit River (797.23 MPN/100 mL) was lower than that in the Mooi River (1126.56 MPN/100 mL), both rivers exceeded the recommended thresholds for irrigation, livestock watering, and recreational use (according to Table A.1).

4.2.1.2.2 Membrane filtration microbial technique

The membrane filtration method (MFM) was employed to quantify bacterial indicator organisms, including total coliforms and *E. coli*, in the Setumo Dam. Table A.2 summarises the results. Sites S1 to S3, located near the WWTP, exhibited total coliform counts that were too numerous to count (TNTC), defined as exceeding 300 colony-forming units (CFU) per 100 mL. The lowest total coliform count (75 CFU/100 mL) was observed at Site S4, closest to the drinking water treatment plant (DWTP). The average total coliform count across the Setumo Dam was 243.75 ± 112.50 CFU/100 mL. *E. coli* levels in the Setumo Dam followed a similar trend to total coliforms. Sites S1 to S3, located downstream of the WWTP, exhibited TNTC *E. coli* levels. The lowest *E. coli* count (73 CFU/100 mL) was recorded at Site S4. The average *E. coli* level in the Setumo Dam was 243.25 ± 113.50 CFU/100 mL, far exceeding the acceptable threshold for irrigation and recreational use, as indicated in Table A.1. Figure 4.6 illustrates the microbiological parameters across the four sites in the Setumo Dam. Site S1, nearest to the WWTP, showed the highest contamination, reflecting the influence of untreated wastewater discharge. In contrast, Site S4, located closest to the DWTP, exhibited the lowest contamination levels, underscoring the role of water treatment processes in reducing microbial loads.

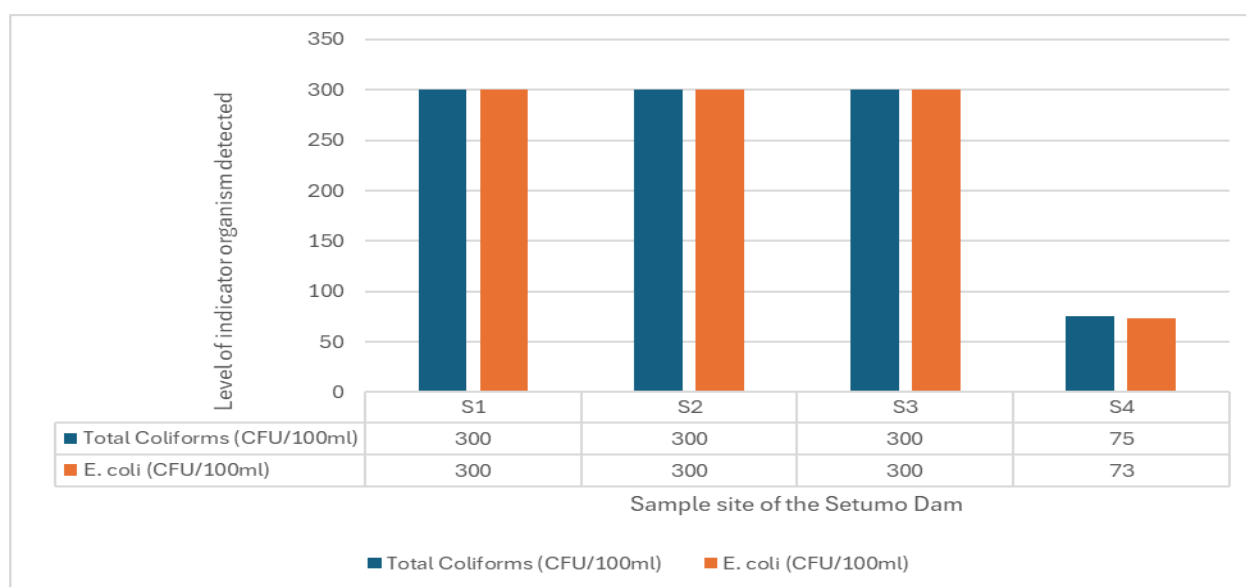


Figure 4.6: Bar chart of microbiological parameters measured across the 4 sites in the Setumo Dam.

Site 1 is nearest to the WWTP and Site 4 is closest to the DWTP.

4.2.1.3 Water quality index

Table 4.1 highlights the sampling sites from which the 34 potentially pathogenic Enterobacteriaceae isolates were obtained and their respective water quality indices (WQI). Most sites complied with the TWQR for pH, except for Sites 2, 3, and 4 in the Setumo Dam where pH values exceeded acceptable ranges. TDS levels at all sites exceeded the TWQR for irrigation and livestock watering, with the Setumo Dam exhibiting the highest TDS values. The *E. coli* levels at all sites were significantly higher than the strictest TWQR threshold of less than 1 count (count/100 mL), indicating substantial faecal contamination. At most of the sites, the *E. coli* levels exceeded 1 000 MPN or CFU/100 ml as described for agricultural use and recommended as acceptable wastewater effluent. Based on these three parameters (pH, TDS, and *E. coli*), the water quality at all sites was categorised as poor.

4.3 Phenotypic characteristics

Determining the phenotypic characteristics is important, as it aids in the identification and understanding of microbial traits associated with virulence, AR, and pathogenic potential. The detection of extracellular enzyme production and extended-spectrum β -lactamase (ESBL) activity, combined with antibiotic susceptibility testing, offers critical data for public health risk assessment and informs water quality management strategies. Additionally, including isolates with atypical colony morphology, presumed to be Enterobacteriaceae, highlights the adaptability of this method in identifying diverse pathogens. This comprehensive phenotypic profiling enhances our understanding of microbial threats in aquatic environments and supports effective interventions to mitigate the risks posed by pathogenic Enterobacteriaceae.

4.3.1 In pursuit of *E. coli* O157

The recovery of presumptive *Escherichia coli* O157 colonies across the three surface water systems using different methodologies is summarised in Table 4.2. A total of 3532 *E. coli* isolates were analysed, from which 818 presumptive *E. coli* O157 colonies were recovered on CT-SMAC agar. Among the methodologies used, immunomagnetic separation (IMS) demonstrated the highest recovery rates, with 254 colonies isolated from the Mooi River and 369 from the Schoonspruit River. In comparison, the defined substrate technology (DST) and membrane filtration technique (MFT) yielded significantly lower recovery rates, with DST recovering 72 colonies from the Mooi River and 32 from the Schoonspruit River, and MFT recovering only 91 colonies from the Setumo Dam. These results highlight the superior specificity and efficiency of IMS in isolating *E. coli* O157.

Statistical analysis of the recovery rates showed a moderate positive Pearson's correlation coefficient (0.61) between *E. coli* O157 recovery from total *E. coli* isolated. However, the p-value of 0.27 indicated no statistically significant association. A one-way ANOVA comparing MFT and DST yielded an F-statistic of 107.22 with a p-value of 0.06, suggesting only marginal differences between the MFT and DST methods used to recover *E. coli* O157, combined with culturing on CT-SMAC agar. These findings suggest the variability in recovery efficiencies across methodologies and the need to prioritise more targeted techniques, such as IMS, for reliable detection of *E. coli* O157 in surface water systems.

4.3.2 Selection criteria of the 34 Enterobacteriaceae isolates

Thirty-four Enterobacteriaceae isolates were selected to represent a diverse cross-section of water sources with poor water quality indices (WQI), as shown in Table 4.1. The selection process aimed to include isolates from various sampling sites to reflect the extent of potential pathogenic contamination in surface water sources. All 34 selected isolates were characterised as putative *E. coli* O157 on CT-SMAC agar and could lyse erythrocytes (α -haemolysin). This subset comprised 12 presumptive *E. coli* O157 isolates confirmed via latex agglutination, 28 isolates exhibiting a green metallic sheen on EMB agar, and six isolates displaying pink or purple colonies. Most (59%) of isolates originated from the Mooi River (Site 6 at Trimpark), a location frequently used for recreation and as a refuge for the destitute, emphasising its vulnerability to contamination. Additional isolates were collected from Schoonspruit River (12%) downstream of a WWTP, Wonderfonteinspruit (9%) downstream of another WWTP, and the Setumo Dam (12%) across four distinct sites.

Table 4.1: Representation of sampling sites harbouring the 34 Enterobacteriaceae isolates

Surface Water Source	Site Description	Water Quality Index (pH, TDS & <i>E. coli</i>)	No. and % of Enterobacteriaceae Selected
Setumo Dam – Site 1	Site close to the WWTP	Poor WQI	1 (3%)
Setumo Dam – Site 2	Distribution point 1	Poor WQI	1 (3%)
Setumo Dam – Site 3	Distribution point 2	Poor WQI	1 (3%)
Setumo Dam – Site 4	Site close to DWTP	Poor WQI	1 (3%)
Schoonspruit River – Site 2	Bodenstein	Poor WQI	2 (6%)
Schoonspruit River – Site 6	Downstream WWTP	Poor WQI	4 (12%)
Mooi River – Site 6	Trimpark	Poor WQI	20 (59%)
Mooi River – Site 7	Pedestrian bridge	Poor WQI	1 (3%)
Wonderfonteinspruit – Site 1	Downstream of WWTP	Poor WQI	3 (9%)

Table 4.2: Recovery of *E. coli* O157 from *E. coli* isolates across the three surface water systems using three methodologies

Methodology	Surface Water	No. of Sampling Sites	Selected No. of <i>E. coli</i>	No. of CT-SMAC positive <i>E. coli</i> O157
MFT	Setumo Dam	4	361	91
DST	Mooi River	10	798	72
	Schoonspruit River	6	450	32
IMS	Mooi River	10	1126	254
	Schoonspruit River	6	797	369
Total <i>E. coli</i> counts			3532	818
Pearson's correlation				0.61
p-value				0.27
ANOVA MFT vs DST (F-Statistics)				107.22
p-value				0.06

The presumptive *E. coli* O157 percentage recovered on Sorbitol MacConkey agar infused with cefixime and potassium tellurite (CT-SMAC). Methodologies: MFT – membrane filtration technique; DST – defined substrate technology; and IMS – immunomagnetic separation. In the case of MFT-selected colonies and DST-selected wells, spot inoculation was used to determine the percentage of *E. coli* O157 recovered on CT-SMAC.

4.3.3 Extracellular and extended spectrum- β -lactamase enzyme producing Enterobacteriaceae

The production of extracellular enzymes by 34 Enterobacteriaceae isolates from three surface water systems (Setumo Dam, Schoonspruit River, and Mooi River) was assessed and summarised in Table 4.3. The selected highlighted values reflect the occurrences of extracellular enzyme production, some at high occurrences and others at low occurrences, indicating the potential of these isolates to cause disease. All isolates demonstrated 100% production of haemolysin, β -galactosidase, and DNase across all sites. Catalase was also highly prevalent, with an overall production rate of 97%, slightly lower at 96% for the Mooi River isolates. Distinct variations were observed in the production of other enzymes. Proteinase production was highest in Schoonspruit River isolates (83%), followed by the Mooi River (75%) and Setumo Dam (50%), resulting in an overall production rate of 71%. Lipase and lecithinase production were also prominent, at 74% and 68%, respectively, with Setumo Dam isolates achieving 100% lipase production. In contrast, gelatinase was the least produced of these enzymes, with an overall prevalence of 65%, despite high production levels in Schoonspruit River isolates (83%). The β -glucuronidase enzyme showed the lowest overall production rate (3%) and was detected in only one isolate (4%) from the Mooi River; this is isolate number 35 (WF1_5). Interestingly, the Mooi River isolates were the only group to produce all 11 tested enzymes, including extended-spectrum β -lactamase (ESBL), which was absent from Schoonspruit River isolates but present in 75% of isolates from both the Setumo Dam and Mooi River.

Table 4.3: Extracellular and ESBL enzyme production of the Enterobacteriaceae isolates

Enzymes produced	Setumo Dam (n=4)	Schoonspruit River (n=6)	Mooi River (n=24)	Total (n=34)
Haemolysin (α)	100% (4/4)	100% (6/6)	100% (24/24)	100% (34/34)
β -galactosidase	100% (4/4)	100% (6/6)	100% (24/24)	100% (34/34)
β -glucuronidase	0% (0/4)	0% (0/6)	4% (1/24)	3% (1/34)
Catalase	100% (4/4)	100% (6/6)	96% (23/24)	97% (33/34)
Proteinase	50% (2/4)	83% (5/6)	75% (18/24)	71% (24/34)
DNase	100% (4/4)	100% (6/6)	100% (24/24)	100% (34/34)
Lipase	100% (4/4)	83% (5/6)	67% (16/24)	74% (25/34)
Gelatinase	25% (1/4)	83% (5/6)	67% (16/24)	65% (22/34)
Lecithinase	25% (1/4)	83% (5/6)	71% (17/24)	68% (23/34)

Enzymes produced	Setumo Dam (n=4)	Schoonspruit River (n=6)	Mooi River (n=24)	Total (n=34)
ESBL	75% (3/4)	0% (0/6)	75% (18/24)	62% (21/34)

4.3.4 Antibiotic phenotype of the thirty-four isolates

Figure 4.9 illustrates the AR phenotype of the 34 Enterobacteriaceae isolates as a heatmap generated using the disc diffusion method (DDM), also known as the Kirby-Bauer method. The darker blocks show resistance, and the lighter blocks show susceptibility of the associated Enterobacteriaceae isolate against a given antibiotic. The isolates were tested against 14 antibiotics spanning nine different antibiotic classes, revealing a diverse range of resistance and susceptibility profiles. The isolates demonstrated complete susceptibility to gentamicin (100%), high susceptibility to ciprofloxacin and chloramphenicol, and moderate susceptibility to trimethoprim. However, universal resistance was observed against sulphamethoxazole and erythromycin, except for strain 35 and strain 28, respectively. Similarly, all isolates resisted amoxicillin and cefazolin, except for strains 35 and 17. Resistance to ampicillin was prevalent, with only five isolates (35, 10, 17, 38, 12) showing susceptibility.

Co-resistance to trimethoprim and sulphamethoxazole was identified in seven isolates (10, 17, 34, 38, 12, 16, and 27) originating from the Schoonspruit and Mooi River water systems. These water systems also accounted for strains resistant to nalidixic acid (35, 19, 9, and 25) and select strains resistant to streptomycin, tetracycline, and kanamycin. Notably, the Mooi River system yielded five strains (31, 16, 22, 23, and 27) resistant to neomycin, highlighting this system's higher prevalence of MDR phenotypes. Among the isolates, strain 34 (Mooi WF1.18) displayed the most extensive resistance phenotype, with resistance to 10 of the 14 antibiotics tested, including streptomycin, tetracycline, erythromycin, amoxicillin, chloramphenicol, trimethoprim, nalidixic acid, and sulphamethoxazole. Conversely, strain 35 exhibited the least resistance, resistant only to erythromycin and nalidixic acid. Interestingly, both strains originated from the Mooi River system, underscoring its role as a reservoir for antibiotic-resistant Enterobacteriaceae.

4.3.5 The biochemical characteristics of the thirty-four strains

The biochemical profiles of the 34 Enterobacteriaceae isolates, summarised in Table 4.4, revealed distinct patterns of metabolic activity based on 14 tested biochemical reactions. The green shading was applied to the table to show which reactions were calculated to get the five coded bio-codes. Bio-code was interpreted using the manufacturer's guide in section 3.5.7. All strains demonstrated the ability to hydrolyse urea, while dulcitol fermentation and phenylalanine

deamination were absent from all isolates. Interestingly, all isolates except strain 32 could use citrate as a carbon and energy source. Consistent with the selection criteria of SMAC-CT agar, most strains could not ferment sorbitol, except strain 28, which exhibited positive sorbitol fermentation. Similarly, adonitol fermentation was rare, being observed only in strain 9. Notably, strains 9 and 35 tested positive for indole production, indicating their ability to convert tryptophan into indole. Glucose fermentation was a common trait accompanied by gas production, highlighting a shared metabolic characteristic across isolates.

Hydrogen sulphide (H₂S) production was observed in seven strains (5, 19, 25, 26, 31, 35, and 27), as indicated by the characteristic black colour change in the reaction wells. Positive reactions were also prevalent for lysine and ornithine decarboxylation and lactose and arabinose fermentation. However, patterns of negative reactions for adonitol, lactose, arabinose, sorbitol, dulcitol fermentation, and phenylalanine deamination were consistent among Setumo Dam isolates. The biochemical profiles varied across water systems. Strains from the Schoonspruit and Mooi River systems (e.g., 26, 13, 16, 17, and 35) displayed positive reactions for up to eight of the 14 biochemical tests. In contrast, the Setumo Dam strains demonstrated up to seven positive reactions at most. Strain 38, sourced from the Schoonspruit River, was unique in its biochemical profile, showing positive reactions only for urea hydrolysis and citrate utilisation, deviating significantly from the rest of the isolates.

Biochemical reaction patterns, visualised with enteropluri codes in Table 4.4, enabled phenotypic identification of the isolates. Approximately 50% of the isolates were identified as *Enterobacter* species, 18% as *Serratia marcescens*, 5% as *Acinetobacter iwoffii*, and 5% as *Citrobacter freundii*. However, 50% of the strains could not be identified and were classified as "unidentified" based on their biochemical profiles. Strain 34, identified as *Enterobacter cloacae*, exhibited an enteropluri code of 22143 and demonstrated broad metabolic activity across the biochemical tests. The metabolic diversity among the strains, particularly those from the Mooi River system, underscores the heterogeneity of Enterobacteriaceae populations in surface water systems and highlights their varying ecological and public health significance. The unique profiles of strains such as 38 and 34 further emphasise the need for detailed characterisation to understand microbial community dynamics.

Table 4.4: Biochemical characteristics of the 34 Enterobacteriaceae strains with the accompanying phenotypic identification Bio-code

Sample ID	Strain	Biochemical ID	Glu 2	Gas 1	Lys 4	Orn 2	H2S 1	Ind 4	Ado 2	Lac 1	Ara 4	Sor 2	Dul 1	PA 4	Urea 2	Cit 1	Bio-Code (without VP)
S2_R1.2	5	Unidentified	+	-	+	+	+	-	-	-	-	-	-	-	+	+	27003
S3_R1.1	6	Unidentified	+	+	+	+	-	+	-	-	-	-	-	-	+	+	36403
S1_R1.1	7	<i>S. marcescens</i>	+	-	+	+	-	-	-	-	-	-	-	-	+	+	26003
S4_R1.1_2	8	<i>S. marcescens</i>	+	+	+	+	-	-	-	-	-	-	-	-	+	+	36003
Sch6_27	9	Unidentified	+	+	-	-	-	-	+	+	+	-	-	-	+	+	30343
Sch6_29	10	Unidentified	+	+	-	-	-	-	-	-	+	-	-	-	+	+	30043
Sch2.1_21	11	Unidentified	+	+	-	-	-	-	-	+	+	-	-	-	+	+	30143
Sch6_31	12	Unidentified	+	+	-	-	-	-	-	-	+	-	-	-	+	+	30043
Sch2.1_19	13	Unidentified	+	+	-	+	+	-	-	+	+	-	-	-	+	+	33143
Sch6_31_2	38	<i>A. iwoffii</i>	-	-	-	-	-	-	-	-	-	-	-	-	+	+	00003
MR7_4	14	<i>E. gergoviae</i>	+	+	+	+	-	-	-	-	+	-	-	-	+	+	36043
MR6_12	15	<i>E. cloacae</i>	+	+	-	+	-	-	-	+	+	-	-	-	+	+	32143
MR6_27	16	<i>E. gergoviae</i>	+	+	+	+	-	-	-	+	+	-	-	-	+	+	36143
MR6_9	17	<i>E. gergoviae</i>	+	+	+	+	-	-	-	+	+	-	-	-	+	+	36143
MR6_8	18	Unidentified	+	+	-	+	-	-	-	-	+	-	-	-	+	+	32043
MR6_33	19	<i>E. cloacae</i>	+	+	-	+	-	-	-	+	+	-	-	-	+	+	32143
MR6_19	20	<i>E. cloacae</i>	+	-	-	+	-	-	-	+	+	-	-	-	+	+	22143
MR6_1	21	Unidentified	+	+	-	-	-	-	-	+	+	-	-	-	+	+	30143
MR6_6	22	Unidentified	+	+	-	-	-	-	-	+	+	-	-	-	+	+	30143
MR6_48	23	<i>E. cloacae</i>	+	+	-	+	-	-	-	+	+	-	-	-	+	+	32143
MR6_8_2	24	Unidentified	+	+	-	-	-	-	-	+	+	-	-	-	+	+	30143
MR6_22	25	<i>C. freundii</i>	+	+	-	-	+	-	-	+	+	-	-	-	+	+	31143
MR6_3	26	Unidentified	+	+	+	+	+	-	-	-	-	-	-	-	+	+	37043
MR6_11	28	Unidentified	-	-	+	+	-	-	-	-	+	+	-	-	+	+	06063
MR6_16	29	<i>E. gergoviae</i>	+	+	+	+	-	-	-	-	+	-	-	-	+	+	36043
MR6_10	30	<i>E. gergoviae</i>	+	+	+	+	-	-	-	-	+	-	-	-	+	+	36043
MR6_32	37	<i>E. gergoviae</i>	+	+	+	+	-	-	-	-	+	-	-	-	+	+	36043
MR6_20	32	Unidentified	-	-	+	+	-	-	-	-	+	-	-	-	+	-	06042
MR6_15	33	<i>E. gergoviae</i>	+	-	+	+	-	-	-	-	+	-	-	-	+	+	26043
MR6_21	36	<i>S. marcescens</i>	+	+	-	+	-	-	-	-	-	-	-	-	+	+	36003
MR6_32_2	31	Unidentified	+	-	-	-	+	-	-	-	-	-	-	-	+	+	21003
WF1_5	35	Unidentified	+	+	+	-	+	+	-	+	-	-	-	-	+	+	35503
WF1_42	27	Unidentified	-	-	+	+	+	-	-	-	+	-	-	-	+	+	07043
WF1_18	34	<i>E. cloacae</i>	+	+	-	+	-	-	-	+	+	-	-	-	+	+	22143

Glu=Glucose; Gas=Gas; Lys=Lysine; Orn=Ornithine; H2S=Hydrogen Sulide; Ind=Indole; Ado=Adonitol; Lac=Lactose; Ara=Arabinose; Sor=Sorbitol; Dul=Dulcitol; PA=Phenylalanine; Cit=Citrate; +: Positive; -: Negative

4.3.6 Genotypic identification and characteristics

The genotypic identification and characteristics of the 34 Enterobacteriaceae were determined using good-quality DNA to conduct PCR, gel electrophoresis and Sanger sequencing. The ARGs and virulence genes profile was also determined using PCR analysis.

4.3.6.1 16S rRNA gene Sanger sequencing identification

Genotypic characterisation of the 34 isolates was conducted using molecular biology techniques, including total DNA extraction, PCR amplification of the 16S rRNA gene, gel electrophoresis, and Sanger sequencing. High-quality DNA was successfully extracted, with A260/A280 ratios ranging from 1.7 to 2.0 and concentrations of ~50 ng/μL, ensuring suitability for downstream analyses. PCR amplification yielded 1500 bp amplicons for all isolates, as confirmed by agarose gel electrophoresis (Figure 4.7). The no-template control (NTC) exhibited no amplification, confirming the absence of contamination and validating the PCR protocol. Strains 36, 27, and 38 were analysed separately, and their amplicons were consistent with the expected size of 1500 bp.

Post-PCR clean-up was performed, and Sanger sequencing of both forward and reverse reads produced high-quality chromatograms with read lengths between 300 and 500 bp, as described by Chen et al. (2014). The sequence data were processed using BioEdit version 7.2.5, where chromatograms were trimmed, reverse-complemented, and concatenated. These concatenated sequences were submitted to the NCBI BLAST platform to identify regions of local similarity, with matches exceeding 90% identity. The sequences were also deposited into the NCBI GenBank database and assigned accession numbers (Table A.5).

Figure 4.8 is a pie chart that illustrates the results that identified all isolates as members of the Enterobacteriaceae family, with species assignments including *Citrobacter freundii* (44%), *Rahnella* spp. (17%), *Enterobacter* spp. (12%), *Serratia* spp., *Hafnia alvei*, *Klebsiella* spp., and *Escherichia coli*. The dominance of *Citrobacter* spp. highlights its prevalence in the sampled surface water systems.

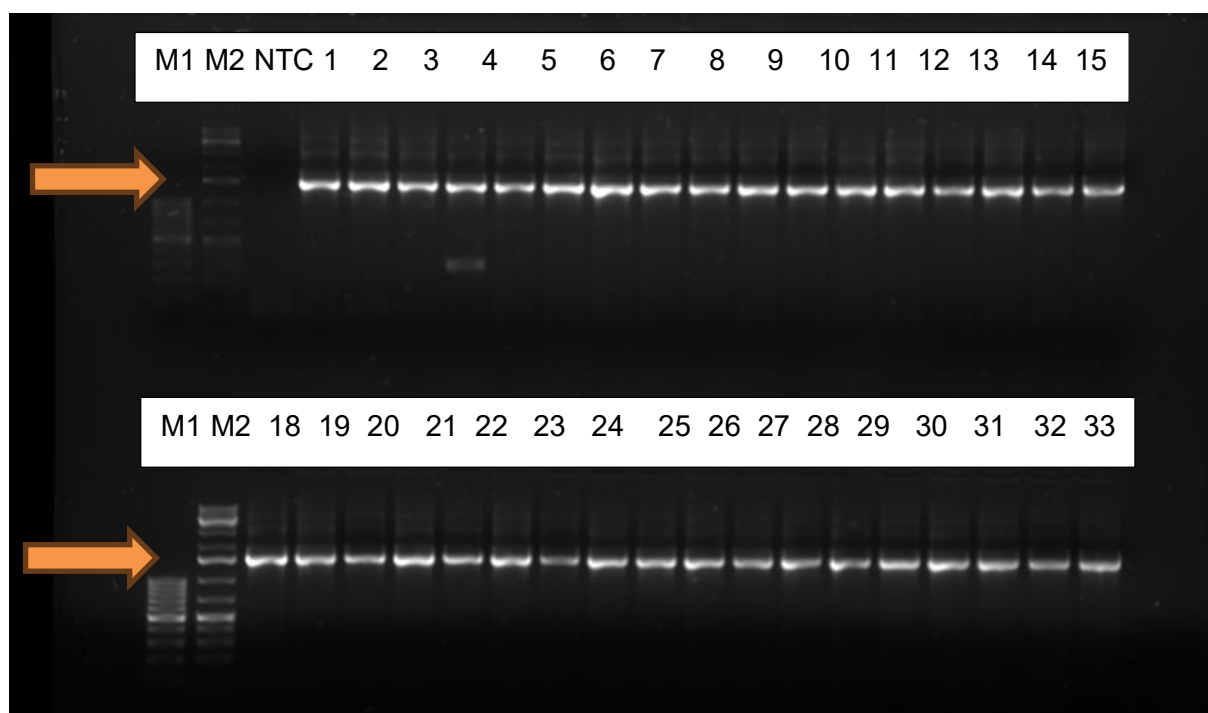


Figure 4.7: A 1.5% (w/v) agarose gel indicating the 16S rRNA gene amplicon with a size of 1500 bp indicated with the blue arrows.

The lane indicated with M1 is a 100 bp molecular weight marker, and the lane marked with M2 is a 1 kb molecular weight marker.

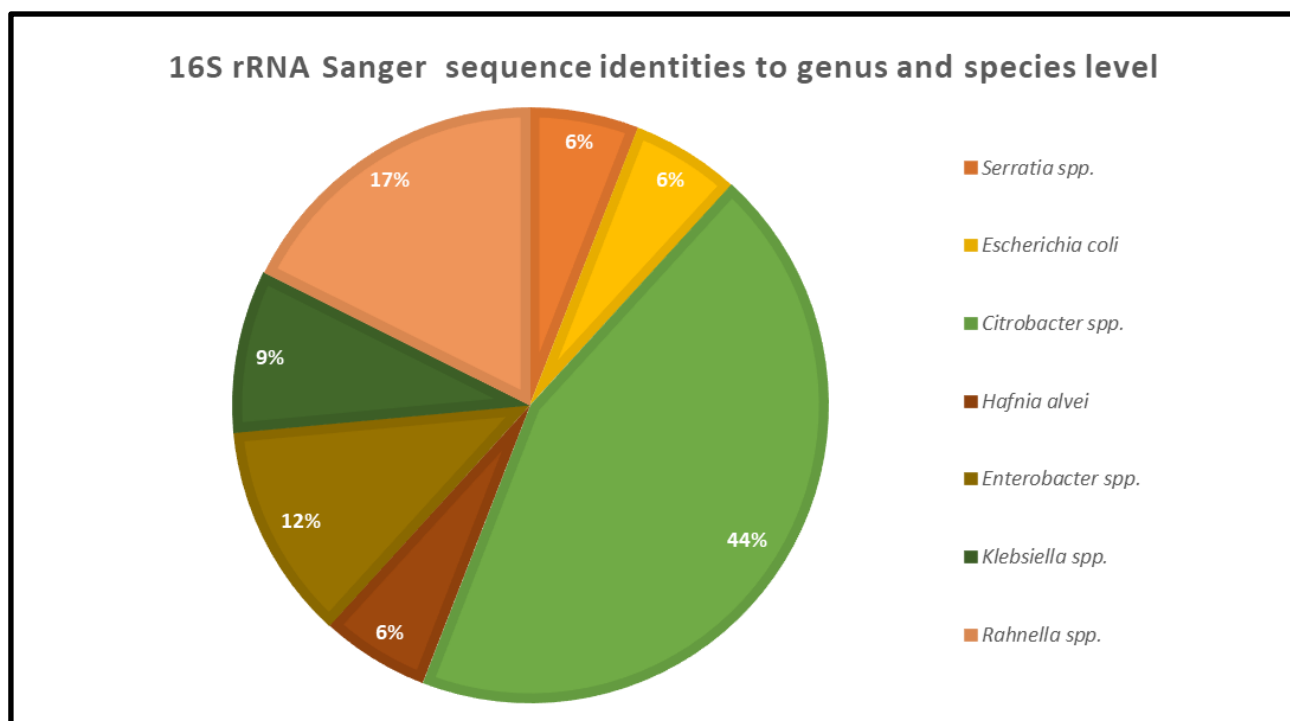


Figure 4.8: The genus and species identities generated from 16S rRNA Sanger sequence analyses.

4.3.6.2 Phylogenetic tree of isolates with reference strains

Figure 4.9 illustrates a maximum parsimony phylogenetic tree and reveals the evolutionary relationships among Enterobacteriaceae isolates collected during the rainy season 2017. The tree incorporates study isolates and reference sequences from the NCBI GenBank database, with *Clostridium perfringens* ATCC 13124 (NR121697.2) as an outlier to establish evolutionary divergence. The analysis revealed two major clusters, with strain 21 (Mooi 6.1) forming a distinct branch, highlighting its genetic divergence from the remaining 33 isolates. These 33 isolates formed a single robust cluster with a high bootstrap value of 100%, indicating substantial evolutionary similarity.

In the primary cluster, three distinct subclusters were identified. The first subcluster comprised strains 6, 19, 20, 30, and 35, closely aligning with *Escherichia coli* and *Enterobacter* reference strains. The second subcluster included strains 8, 9, 10, 11, 17, 29, 31, 32, 33, 34, and 38, which were evolutionarily similar to *Serratia spp.* and *Hafnia alvei*. The third and largest subcluster consisted of strains 7, 12, 13, 15, 16, 18, 22, 23, 24, 25, 26, 27, 28, and 37, exhibiting close similarity to *Citrobacter freundii* and *Klebsiella spp.* This dominance of *Citrobacter freundii* reflects its prevalence in the sampled aquatic ecosystems.

Strain 36 (WF1.42) formed an independent branch outside the major clusters, suggesting unique genetic characteristics or divergence from other Enterobacteriaceae members. The distinct clustering patterns observed in the phylogenetic tree highlight the genetic diversity of the isolates, with significant representation of *Citrobacter freundii*, *Rahnella spp.*, and other genera. The evolutionary relationships outlined in this analysis underscore the ecological complexity of the sampled surface water systems and provide valuable insights into the adaptability and potential pathogenicity of Enterobacteriaceae species in these environments.

4.3.6.3 Antibiotic-resistant genotype of the thirty-four Enterobacteriaceae

Figure 4.9 comprehensively represents the phylogenetic relationships, AR phenotypes, and the presence or absence of ARGs among the 34 Enterobacteriaceae isolates. The analysis screened for nine ARGs, revealing varying prevalence patterns across the isolates. The *int11* gene, a marker of anthropogenic pollution and a key integron-associated ARG, was the most abundant in 79% of the strains. This was followed by the *AmpC* gene (76%), *sul2* (44%), *sul1* (38%), *blaNDM* (29%), *blaTEM* (20%), *blaCTX* (11%), *blaOXA* (8%), and *blaKPC* (8%). Notably, *int11* was absent in strains 8, 18, 21, 25, 29, 32, and 38, indicating potential environmental or genetic variability in ARG distribution.

The heatmap further reveals that several strains (5, 7, 28, 29, 30, 31, 32, and 38) did not express the *AmpC* gene, a marker commonly associated with β -lactamase resistance. Strains 5, 6, 9, 10, 11, 12, 13, 14, 16, 26, 27, 33, 36, and 37 expressed four of the nine ARGs, highlighting their MDR potential. Four strains (10, 12, 37, and 38) simultaneously expressed *sul1* and *sul2*, genes associated with sulphonamide resistance, suggesting significant selective pressure for these ARGs in the sampled environments.

Extended spectrum β -lactamase (ESBL) genes were also investigated. Strains 5, 6, 8, 14, 16, 18, 26, 27, and 29 expressed *blaTEM* and/or *blaCTX*, indicating the ability to hydrolyse a broad spectrum of β -lactam antibiotics. Furthermore, carbapenem resistance was detected in strains 6, 8, 9, 11, 13, 22, 24, 27, 28, 29, 30, 31, 32, 33, and 36, reflecting a concerning prevalence of resistance to this critical antibiotic class. Four strains (6, 8, 27, and 29) expressed ESBL and carbapenem resistance genes, highlighting their heightened potential for clinical and environmental significance.

The least ARG diversity was observed in strains 7, 18, 21, 25, 32, and 38, expressing only two ARGs. This contrasts with the broader ARG profiles, emphasising Enterobacteriaceae's genetic variability. The findings from Figure 4.9 emphasise the widespread distribution of ARGs among environmental isolates, particularly genes linked to anthropogenic pollution (*int1*), β -lactamase (*AmpC*), and sulphonamide resistance (*sul1* and *sul2*). These results highlight the urgent need to monitor and mitigate the dissemination of ARGs in aquatic ecosystems.

4.3.6.4 Housekeeping and virulence genes

The prevalence of housekeeping and virulence genes in the 34 Enterobacteriaceae strains is summarised in Table 4.5. The housekeeping gene *uidA*, encoding the β -glucuronidase (GUS) enzyme, was universally expressed across all strains (100%). This result is consistent with the functional conservation of *uidA* as a key metabolic marker in Enterobacteriaceae.

The primary aim of this study was to isolate *E. coli* O157 strains, prompting the investigation of Shiga toxin (*stx1* and *stx2*) genes. However, neither *stx1* nor *stx2* was detected in any of the isolates, aligning with the absence of *E. coli* O157 as confirmed by Sanger sequencing. This absence of *stx* genes highlights the genetic variability within the sampled Enterobacteriaceae population. It confirms that none of the strains exhibited the mark of a virulence factor of *E. coli* O157.

Table 4.5: Prevalence of housekeeping and virulence genes from the selected surface water systems.

Virulence gene(s)	<i>E. coli</i> O157 ATCC 700728	<i>E. coli</i> ATCC 10536	No. of isolates with indicated virulence gene(s)			
			Setumo Dam	Schoonspruit River	Mooi River	Total of 34 Isolates (%)
<i>uid_A</i>	+	+	4	6	24	100%
<i>stx1</i>	-	-	0	0	0	0%
<i>stx2</i>	-	-	0	0	0	0%
<i>eae</i>	+	-	1	1	5	20%
<i>hlyA</i>	+	-	2	3	12	50%

Interestingly, only 50% of the isolates harboured the *hlyA* gene, which encodes haemolysin, a cytolytic toxin associated with increased virulence. This result deviates from the overall haemolytic virulent phenotype observed. This is a common finding in research, since hemolytic phenotype could be related to other *hly* genes than the one used in this study. The intimin gene (*eae*), associated with bacterial adherence to host epithelial cells, was detected in 20% of the isolates. Notably, the highest prevalence of virulence genes, including *hlyA* and *eae*, was observed in isolates from the Mooi River, highlighting its potential as a reservoir for pathogenic strains. These findings highlight the variability in virulence gene profiles across the sampled surface water systems. While the absence of *stx* genes indicates that none of the isolates are Shiga toxin-producing, the presence of *hlyA* and *eae* in several isolates suggests a risk potential for virulence. This further emphasises the importance of monitoring these and other associated virulence genes in Enterobacteriaceae isolated from environmental water sources.



4.3.7 Whole genome sequencing and computational molecular biology tools

This section focuses on the whole genome sequence and computational molecular analyses that were performed on the *Citrobacter* species. The phenotypical and genotypical characterisation aided the selection of this genus for several reasons. This included the prevalence of this genus as the most abundant of the Enterobacterial strains with 44% of isolates from all three surface water systems (Setumo Dam, Schoonspruit River and Mooi River). Four *Citrobacter* strains were selected for the following section.

4.3.7.1 Whole genome sequence quality and identity

Table 4.6 presents the sequenced *Citrobacter* genomes, ranging from 62 to 144 contigs. PubMLST analyses confirmed that strains S21 and S23 were *C. braakii*, while S24 and S25 were *C. murlinae* and *C. portucalensis*, respectively. Average nucleotide identity (ANI) analyses validated that S21 and S23 were indeed *C. braakii* (ANI > 98%), while strain S24 showed a 99.26% ANI compared to *C. murlinae* strain CL P080C. Strain S25 was identified as *C. portucalensis* and shared 90% identity with many *C. freundii* genomes. The genome sizes of *C. braakii* strains S21 and S23 were 4.98 Mb and 4.80 Mb, respectively. Strains S24 and S25 contained plasmid replicon types and exceeded 5.2 Mb, indicating they were larger than the *C. braakii* genomes. The GC content of the *Citrobacter* genomes ranged from 50% to 52%. The GC content for *C. braakii* was approximately 52%, consistent with the reference strain *C. braakii* ATCC 8090T. The GC content for *C. murlinae* strain S24 was 50.62%, matching that of strain CL PC080C, while *C. portucalensis* strain S25 had a GC content of 51.49%, aligned with the reference strain *C. portucalensis* A60T.

4.3.7.2 Virulence genes detected in whole genome of the four *Citrobacter* species

The heatmap (Figure 4.10) was developed to illustrate the virulence gene profiles across the four sequenced genomes to identify the primary pathogenicity genes in *C. braakii*, *C. murlinae*, and *C. portucalensis*. This study identified significant virulence factors explained in Table A.5 related to adhesion, biofilm formation, iron acquisition, siderophore movement, environmental stress response, and cell surface localisation in the strains examined. Notably, the genes responsible for cell surface localisation of the CPS (*vexABCDE*) and those involved in capsule polysaccharide biosynthesis (*tviBCDE*) can serve as exclusive markers to differentiate the *C. braakii* genomes from those of *C. murlinae* and *C. portucalensis*. Furthermore, the *C. murlinae* S24 strain exhibited a distinct virulence profile relative to the other analysed genomes. This genome featured the *iroABCDEN* gene cluster, which encodes the heme receptor protein *ShuA* for the iron-salmochelin siderophore system. Importantly, the iron uptake gene *chuY* was found to be unique to this strain.

4.3.7.3 Antibiotic-resistant genes detected in the whole genome

The ARGs (detailed in Table A.6) in four sequenced *Citrobacter* genomes were analysed, revealing a total of 40 genes identified by the CARD. Notably, the *acrAB-TolC* complex, including the transcriptional activator *marA*, was common across all species. Each strain exhibited distinct beta-lactamase genes: S21 (*blaCMY-83*), S23 (*blaCMY-74*), S24 (*blaCMY-59*), and S25 (*blaCMY-77*). The *acrD* efflux pump and *baeR* regulon supported the observed phenotypic resistance to aminoglycosides in all strains. Additionally, *C. portucalensis* S25 harboured the quinolone-resistant gene *qnrB13*, with phenotypic susceptibility to nalidixic acid. Other quinolone resistance genes (*emrAB/TolC*, *patA*, and *mdtHK* efflux complexes) were also detected, linking them to multidrug efflux processes. Most of these variables seemed to be connected to multidrug efflux pump processes, which included genes such as *mdtABCGHK*. The heatmap depicts the fact that *C. braakii* S21 and S23 strains were more similar in their ARG profiles (Figure 4.11) than the others. This was confirmed by the presence of the MDR *mdtF* gene in these two genomes. The heatmap indicated that *C. braakii* strains S21 and S23 shared a similar ARG profile, confirmed by the presence of the *mdtF* gene. Unique to *C. braakii* S21 was the *pmrE* gene, while the *acrF* gene, related to MDR, was found only in *C. murlinae* S24.

Table 4.6: Four *Citrobacter* species were selected and successfully whole genome sequenced

Bacterial strains	<i>Citrobacter braakii</i> S21	<i>Citrobacter braakii</i> S23	<i>Citrobacter murlinae</i> S24	<i>Citrobacter portucalensis</i> S25
Assembly size (bp)	4 987 947	4 805 424	5 481 339	5 269 614
Contigs	144	62	104	91
G+C content	52.12	52.17	50.62	51.49
N ₅₀	60775	145585	192439	154085
PubMLST – species ID	<i>C. braakii</i>	<i>C. braakii</i>	<i>C. murlinae</i>	<i>C. portucalensis</i>
Average nucleotide identity-ANI (%)	<i>C. braakii</i> (98.6)	<i>C. braakii</i> (98.7)	<i>C. murlinae</i> (99.26)	<i>C. portucalensis</i> (98.6)

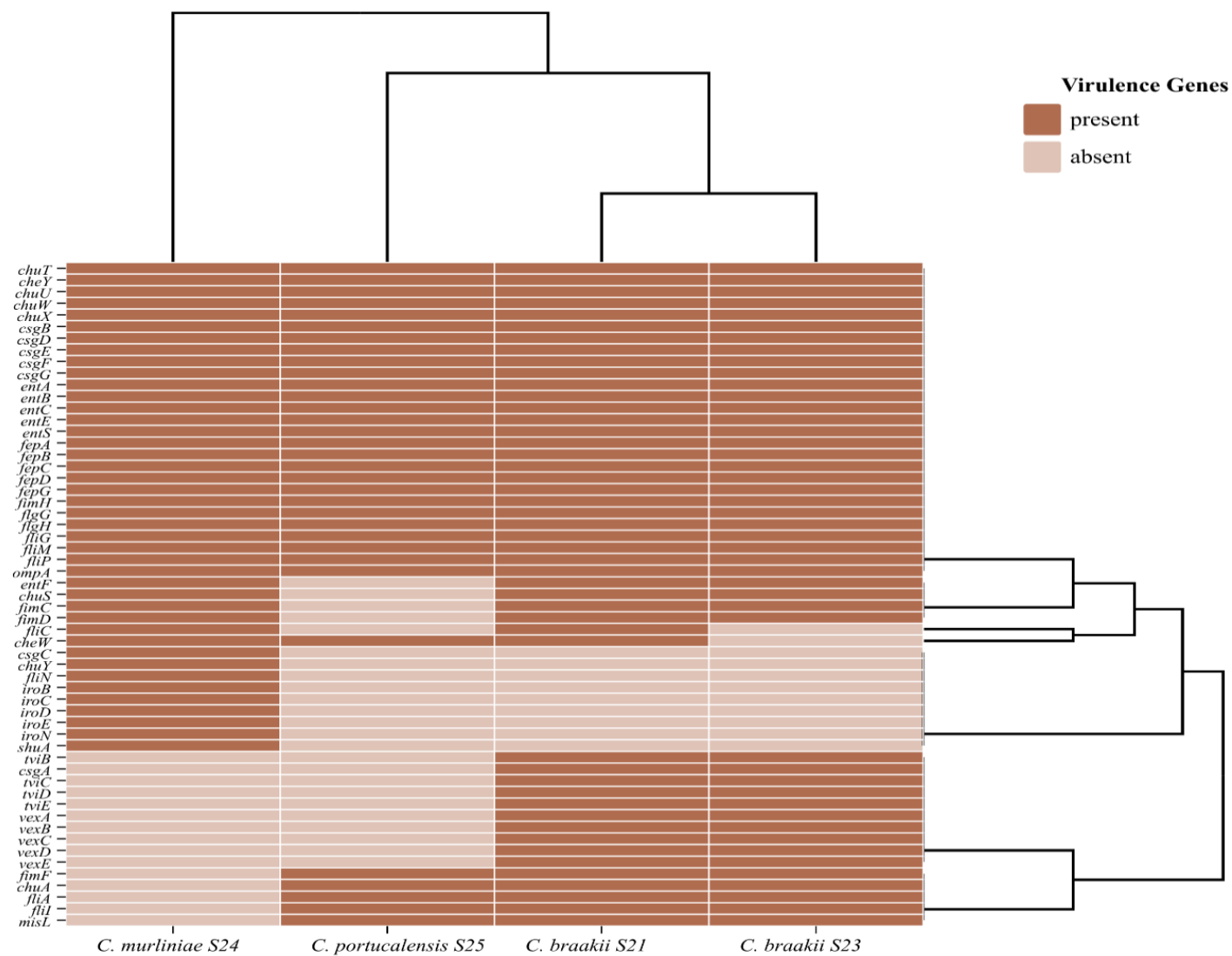


Figure 4.10: Heatmap reflecting the presence and absence of virulence genes (65 in total) across the four sequenced *Citrobacter* species

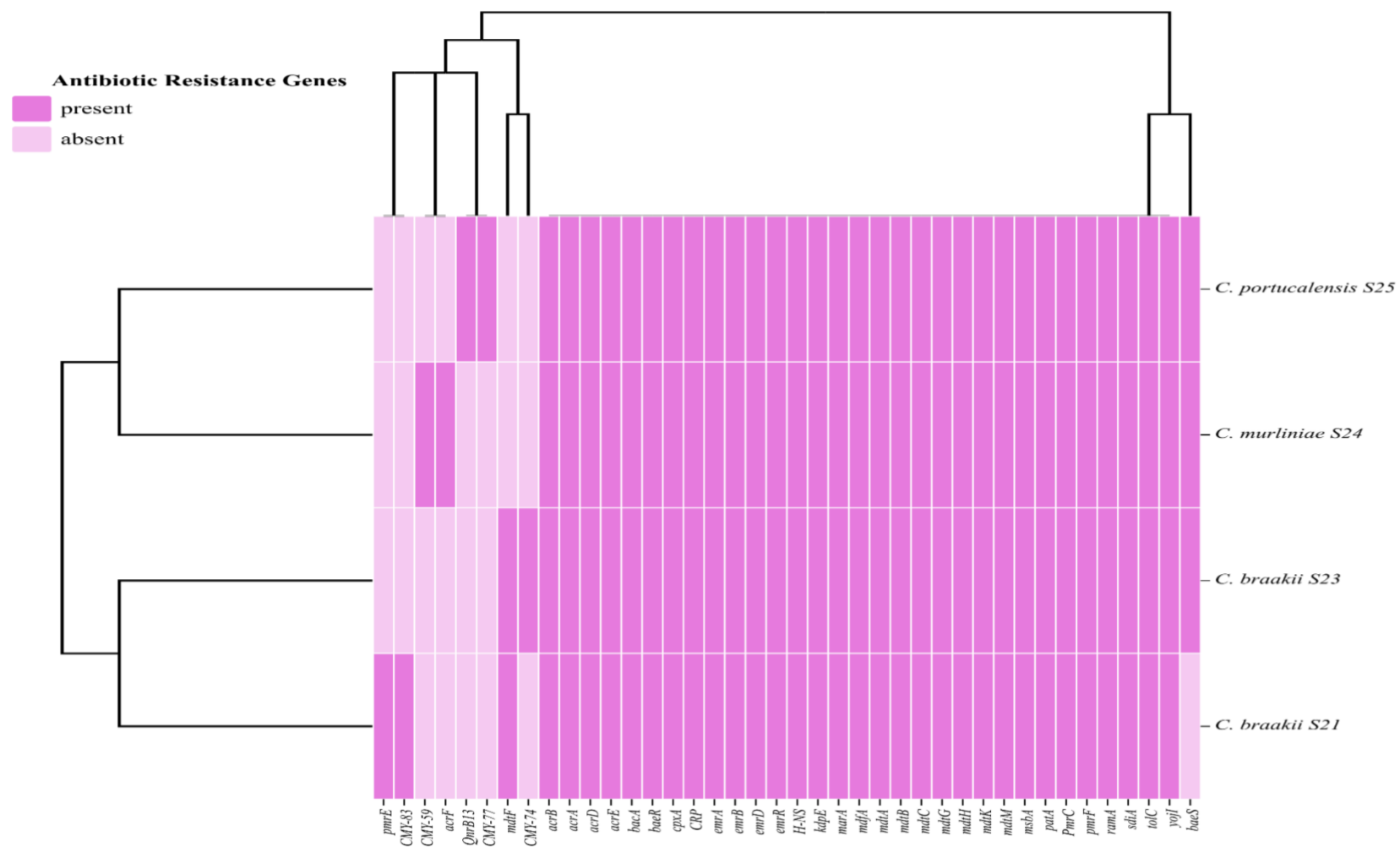


Figure 4.11: Heatmap reflecting the presence and absence of antibiotic-resistant genes (40 in total) across the four sequenced *Citrobacter* species

4.3.7.4 Metabolic pathway

The metabolic pathways data (Figure A1, A2, A3 and A4) support the phenotypic findings and provide an in-depth insight into the *Citrobacter* genomes. The *Citrobacter braakii* strain S21 exhibits active pathways for glycolysis, the TCA cycle, and oxidative phosphorylation, indicating robust energy metabolism. Amino acid biosynthesis pathways such as glutamine and arginine biosynthesis were present, which are crucial for cellular growth and stress response. Lipid metabolism pathways, including fatty acid biosynthesis, suggest membrane stability and adaptation to environmental conditions. The presence of pathways for glycoside hydrolase enzymes suggests the ability to degrade polysaccharides, which may facilitate nutrient acquisition in extracellular environments. Siderophore biosynthesis and secretion pathways are evident, implying an enhanced ability to acquire iron in host environments. Pathways related to quorum sensing suggest communication capabilities for biofilm formation and virulence regulation. Resistance mechanisms such as β -lactamase synthesis and efflux pumps indicate resilience to antibiotics targeting cell wall synthesis or intracellular processes. These findings compare with the phenotypic characterisation and the genes detected in WGS.

The *C. braakii* strain S23 reveals similar patterns, such as active pathways for carbohydrate metabolism, particularly starch and sucrose degradation, pointing to versatility in utilising diverse carbon sources. Lipid degradation pathways, such as β -oxidation, are also active, facilitating energy production. Extracellular lipase and protease pathways are prominent, suggesting the strain's ability to degrade complex substrates in the extracellular environment. Pathways for lipopolysaccharide biosynthesis indicate potential immune evasion capabilities. The presence of phenazine biosynthesis suggests oxidative stress resistance and enhanced virulence. Active efflux pump pathways and aminoglycoside-modifying enzyme synthesis suggest significant resistance to aminoglycoside antibiotics and extended-spectrum β -lactamase activity.

The *C. murlinae* strain S24 exhibits strong activity in amino acid metabolism pathways, particularly for methionine and cysteine, which are critical for sulphur metabolism and stress response. The TCA cycle is prominent, indicating efficient energy generation. Glycosyl hydrolase and cellulase production pathways suggest adaptation to degrading plant polysaccharides in its extracellular environment. Biofilm formation pathways are highly active, supported by quorum sensing and exopolysaccharide biosynthesis. This indicates the strain's ability to form protective communities in host environments. Resistance mechanisms include multidrug efflux pumps and enzymes targeting macrolides, correlating with resistance to broad-spectrum antibiotics.

The *C. portucalensis* demonstrates pathways for energy metabolism, particularly oxidative phosphorylation, and biosynthesis of branched-chain amino acids like leucine and isoleucine,

which are vital for growth and stress resistance. The presence of lipase and protease production pathways suggests efficient extracellular nutrient degradation. Pathways linked to siderophore production and type III secretion systems highlight virulence mechanisms focused on host invasion and nutrient acquisition. β -lactamase and tetracycline resistance pathways were prominent, reflecting a strong capacity to survive in environments with antibiotic pressure.

4.3.8 Comparison between phenotypic and genotypic identification methods

Table A.5 shows the genus and species identity of the 34 isolates. Three methods were used, the first was based on a phenotypic methodology (biochemical identification), thereafter the 16S rRNA gene Sanger sequencing was done, and lastly selected isolates were subjected to next generation sequencing (known to be a targeted and specific identification approach), more specifically WGS. The identities for the phenotypic characterisation yielded 17 identities, where *Enterobacter* species were the most dominant genus. Sanger sequencing revealed the most predominant genus to be *Citrobacter* species. Only selected strains were subjected to WGS, and the identity similarities were above 98% to genomes in the NCBI database. Interestingly, only strain 25 (MO6_22) was consistently identified as the same genus by all the different identification analyses. However, the whole genome sequence-derived identity was more accurate in identifying the species. This is expected since *C. portucalensis* genomes bear a 90% similarity with *C. freundii*. Although biochemical analysis is a quick and inexpensive approach to identification, it is not reliable for genus and species characterisation. With respect to Sanger sequencing, the 16S rRNA gene is a universal bacterial gene target, but it is not target-specific for different genus groupings in the Enterobacteriaceae family. The whole genome sequence approach provides accurate and reliable identification at the species level because the entire genome is considered and not just a fragment. However, if solely used for identification purposes, WGS could be very expensive. Furthermore, when comparing the phenotypic to the WGS identities, it was found that three isolates showed the same genera identities. Whereas, when comparing the 16S rRNA Sanger sequencing to WGS the same five genera was identified with both genotypic approaches.

CHAPTER 5

DISCUSSION

5.1 Introduction

Enterobacteriaceae represents a heterogeneous group of Gram-negative bacteria, usually found in different water bodies, including surface waters. Their presence in these systems indicates faecal contamination and poses significant risks to public health, considering that they can harbour pathogenic varieties. The presence of Enterobacteriaceae in surface water is often related to human activities, such as agricultural runoff and wastewater discharge, which could introduce these microorganisms into aquatic systems (Pal & Jain, 2019; Fadare & Okoh, 2021). Some studies suggested that Enterobacteriaceae, particularly *Escherichia coli*, is a good indicator of water quality with regard to faecal contamination. For instance, Pal and Jain (2019) expressed that the presence of coliform bacteria, such as *E. coli*, in drinking water sources reflected poor water quality and potential health risks. Similarly, Vivas et al. (2019) emphasised that over 50% of waterborne diseases are associated with bacteria from the Enterobacteriaceae family, underscoring their relevance in public health. Their presence in surface water is of concern because the organisms can persist and multiply in aquatic settings, thus increasing the risk of waterborne infections. Such instances have been documented by Cohen et al. (2020) and Abera et al. (2014).

Abiotic factors affecting environmental persistence include temperature, pH, and nutrient conditions. Akinola et al. (2022) established that well water in Nigeria showed high counts of Enterobacteriaceae, indicating its poor microbiological quality, most probably due to the contribution of surface water and prevailing environmental conditions. In contrast, Holguín-Sterling (2023) explains how microbial communities in aquatic environments impact biochemical processes like fermentation, which subsequently influence crop quality. The interaction between environmental determinants and microbial populations underscores the intricate nature of water quality management in both agricultural and urban environments. The issue of AR in Enterobacteriaceae found in surface waters has emerged as a significant concern. According to the findings of Cohen et al. (2020), MDR variants of Enterobacteriaceae have been identified in coastal waters, which raises concern about their possible effects on human health.

The existence of extended-spectrum β -lactamases (ESBL) and carbapenem-resistant Enterobacteriaceae in aquatic ecosystems exacerbates the challenges facing public health, given that these resistant organisms may be transferred to humans via contaminated water supplies (Kittinger et al., 2016). Research conducted by Salamandane et al. (2021) supports this concern,

indicating elevated concentrations of antibiotic-resistant Enterobacteriaceae in drinking water in Maputo and Mozambique, which presents considerable danger to the surrounding communities. Apart from serving as markers of faecal pollution, Enterobacteriaceae may also play a significant role in the deterioration of water quality due to their metabolic processes (Gorrasí, 2021). Specifically, research conducted by Velmurugan and Pandian suggests that the occurrence of Enterobacteriaceae can influence water quality parameters, which are essential for evaluating the overall condition of aquatic ecosystems (Velmurugan & Pandian, 2023). Such degradation can lead to further ecological disruptions, which have consequences for human health, other aquatic organisms, and biodiversity in general (Mafany et al., 2021).

The presence of Enterobacteriaceae in surface water has implications that exceed being biohazardous in nature alone. The contamination of water bodies can disrupt the economics of a community, particularly those that rely on agricultural systems and fish farms (Hamza et al., 2020). The research conducted by Fadare and Okoh underscores the significance of wastewater effluents in the propagation of antibiotic-resistant Enterobacteriaceae, which poses a threat to fish populations and, as a result, affects local fisheries adversely (Fadare & Okoh, 2021). Additionally, the financial impact of waterborne diseases attributed to these pathogens may exert considerable pressure on healthcare systems, especially in developing nations with restricted resources (Singh et al., 2020). Strategies aimed at reducing the hazards linked to Enterobacteriaceae in surface water should concentrate on enhancing water management practices alongside improving public health monitoring systems. Establishing robust water treatment and disinfection measures is crucial for decreasing the microbial burden in water sources (Malavi et al., 2021). Furthermore, public health programmes on hygienic practices and the significance of safe water consumption aimed at educating communities may reduce the spread of waterborne pathogens (Malavi et al., 2018).

The incorporation of effective and reliable microbial monitoring systems in water quality evaluations can yield crucial data that could enable policymakers to make well-informed choices on water safety and public health (Kostoglou, 2023). The presence of Enterobacteriaceae in surface water is a multidimensional problem that includes features of public health, environmental sustainability, and economic robustness (Haque et al., 2014). Water quality management with respect to these bacteria requires a multifaceted approach, considering that they are indicators of faecal pollution and a potential source of AR. Further studies and systematic monitoring will be required to understand the behaviour of Enterobacteriaceae in aquatic systems in order to develop effective measures for the mitigation of impacts on human health and the environment. The characteristics of Enterobacteriaceae in surface water are crucial for understanding water quality and public health risks. Therefore, monitoring their presence in surface water helps assess the effectiveness of water treatment processes, detect environmental pollution, and guide

interventions to protect human and ecosystems health. Understanding their survival, distribution, and resistance patterns in water systems is essential for managing water resources.

5.1.1 Water quality

Temperature, pH, and TDS are important metrics in determining water quality. In this study, the temperature for the sampled sites was between 18.60°C and 25.60°C, consistent with previous studies (Mavis et al., 2018; Kruger and Shongwe, 2004). Temperature influences microbial growth in water. Kruger and Mavis et al. (2018) report on temperature trends ranging from 18.18°C to 28.5°C, respectively, from 1960 to 2013. However, a study by Okeke et al. (2022) suggests that even within this range, there might still be concerns for safety as pathogenic bacteria could potentially propagate in these conditions. Their study reported a temperature range of 25.8°C to 31.8°C, which is higher than the findings in this study.

However, the pH range in this study reflects a minimum site reading of 7.67 and a maximum site reading of 9.24. The maximum pH value exceeded the stipulated target water quality range prescribed by DWS (1996). The highly alkaline water poses a risk for eye, skin, ear, and mucous membrane irritation if the water is accidentally swallowed, since this water source is used for recreational purposes (DWS, 1996). The high alkaline water might be explained by agricultural runoff that can lead to the loss of earth elements such as calcium, magnesium, barium, and strontium into surface water bodies. Continuous monitoring of surface water for pH is important as this is a good indicator of environmental stressors (Elrashidi et al., 2007). The average pH values ranged from 8.23 to 8.55, which comply with acceptable ranges and are similar to various studies (Dorairaju et al., 2013; Kumar & Dua, 2010).

TDS levels ranged widely from 327 mg/L to 2197 mg/L, with Setumo Dam exhibiting the highest concentrations. TDS is an indicator of dissolved mineral content in water, and high TDS levels suggest elevated salinity, which poses a challenge for freshwater organisms and may limit the water's suitability for irrigation and livestock watering (Brent et al., 2022, Kunz et al., 2013). Elevated TDS's in water bodies can result from various anthropogenic activities, particularly the discharge of effluents from WWTPs (Mukate et al., 2018; Iloms et al., 2020; Olabode et al., 2020). These effluents introduce a range of minerals and organic materials that alter the natural composition of water, potentially affecting both environmental and human health (Fuentes-Rivas et al., 2020). High TDS levels thus signal increased pollution, influencing water quality and limiting the range of activities for which the water can be used, from recreation to agricultural applications. Monitoring of these parameters is useful in health risk assessment methods to determine the long-term effects of contaminants on surface water bodies that serve as source water for

downstream drinking water, irrigation for crop production, as well as livestock watering (Kaur et al. 2020; Mohammadpour et al. 2022).

The microbial water quality assessment focuses on bacteriological indicators such as TCs as an indicator of the integrity of the water distribution system and *E. coli* as an indicator of faecal contamination (Zagainova et al., 2020; North et al., 2014). This necessitates monitoring efforts of these indicator organisms in surface water to ensure compliance with safety standards and to protect public health (Tambi et al., 2023; Saxena et al., 2015). Total coliforms, including *E. coli*, are essential indicators of water contamination and the potential prevalence of harmful pathogens in these water resources. While total coliforms are not typically considered to be pathogenic, their detection in water sources is a strong indicator of contamination (Aram et al., 2021; Rompre et al., 2002). Inadequate water treatment facilities have led to sanitary deficits, affecting the most vulnerable populations, who often lack access to treated source water and proper waste disposal (Brion et al., 2000; Zeki, 2022; Momba and Mfenyana, 2005). These sanitary deficits may lead to waterborne diseases, such as diarrhoea, cholera, typhoid fever, hepatitis, and intestinal dysfunction (Bertoncini & Cavassin, 2019; Uprety et al., 2020).

This study showed TC and *E. coli* prevalence across the 20 sampled sites at varying levels. The area of the Setumo Dam closer to the WWTP plant showed the highest levels of total coliforms and *E. coli* of 300+ or too numerous to count at S1, S2, and S3 sites. This suggests very high contamination levels, making the water unfit for irrigation and recreational activities. The high levels detected across the 20 sampled sites could be due to the samples being taken during the rainy season. Interestingly, a common trend of high TC and *E. coli* levels was observed downstream from the WWTPs at Site S1 of the Setumo Dam, downstream from the Mmabatho (A) WWTP, Site SCH6 located downstream from the Klerksdorp (B) WWTP that discharges its effluent into the Schoonspruit River, and Site WF1 located downstream from the Merafong (C) WWTP that discharges into the Wonderfonteinspruit, a tributary of the Mooi River.

This is consistent with findings from studies that indicated a link between WWTP non-conformance upstream and poor effluent discharge into water bodies downstream (Suzuki et al., 2019; Adomako et al., 2021; Makuwa et al., 2020). However, Site MR8, which is also located downstream from the WWTP, showed significantly lower levels of *E. coli*. Nevertheless, the low level of *E. coli* was still above the TWQR described by the former DWAF, now DWS. Furthermore, the Green Drop report (DWS, 2022) states that all 10 WWTPs in the NWP show microbial non-compliant effluent discharge levels above 1000 indicator organism counts per 100 ml. This is worrying since the same report only reveals microbial compliant effluent in nine out of a total of 115 WWTP assessed in South Africa. Furthermore, it has been reported that more *E. coli* occurs

in water bodies closer to WWTPs than in water further away, indicating that the treatment process needs improvement (Asian et al., 2018; Mbanga et al, 2020).

In addition to the efficiency of WWTP in close proximity to surface water sources, the incidence of high microbial indicator organisms could be linked to anthropogenic activity. Figure 3.1 maps out the land use surrounding the sampled sites. Along the Mooi River Sites MR5–8, there are high rates of urban formal settlements, several smallholdings, some industrial activity and agricultural activity. Agricultural and recreational activity has been linked to high coliform and *E. coli* levels in other studies (Neill et al., 2018, Ibekwe et al., 2023). The same observation was made according to Figure 3.1 for the Schoonspruit River, with the exception of several mining activities. Large quantities of water are used during extraction and processing of minerals and discharge may carry these indicator organisms (Bosch et al., 2023, Jordaan and Bezuidenhout, 2016). In a study conducted at the Gauhau Phosphorite Mine in China, *E. coli* was prevalent in surface water in close proximity to the mine, confirming our findings (Li et al., 2019).

The high coliform and *E. coli* levels in the Setumo Dam, however, could be driven by urban townships arising along the water source, with questionable sanitary practises due to lack of sanitary infrastructure (Mallin et al., 2000). Studies, for example, have reported elevated concentrations of faecal indicator organisms in urbanised watersheds due to sewage system outfalls and stormwater runoff (Hathaway and Hunt, 2010). Seasonal variations and hydrological events, such as heavy rainfall, can further exacerbate the contamination of surface waters with coliforms and *E. coli* due to increased runoff surrounding human and animal land use (Américo-Pinheiro et al., 2021, Jordaan and Bezuidenhout, 2016). The water quality in the Setumo Dam and connecting rivers makes it very unsafe for human health and requires immediate action to reduce sources of pollution and disease. The information obtained is consistent with the literature concerning water quality degradation due to anthropogenic activities. The existence of strains such as *E. coli* O157 brings pre-existing problems, stressing the need for effective monitoring and management of these ecosystems and their human uses.

5.1.2 Phenotypic characteristics of Enterobacteriaceae isolated from surface water

Characterising the microbial isolates from the Setumo Dam, Mooi River, and Schoonspruit River brought to light important facts concerning the pathogenicity potentiality and the biochemical activity, specifically with respect to Enterobacteriaceae. Characterising Enterobacteriaceae from surface water is essential for understanding their ecological roles, potential health risks, and contributions to AR. Biochemical traits, extracellular enzyme production, and antibiotic phenotypes offer critical insight into the behaviour, pathogenicity, and resilience of these bacteria

in aquatic environments where they serve as indicators of faecal contamination and public health hazards.

The 34 presumptive *E. coli* O157 were isolated on CT-SMAC media, some of which were done in combination with IMS, which is recommended for detecting this pathogen in environmental samples (Onoue et al., 1999, Muller and Ehlers, 2005, Ngwa et al., 2013). This selective media differentiated pathogenic *E. coli* from commensal *E. coli* based on its inability to ferment sorbitol (Anyanwu et al., 2022, Islam et al., 2015). The results gathered from CT-SMAC agar was confirmed with Rainbow O157 chromogenic agar. According to Murase et al. (2012), the presumptive *E. coli* O157 isolates show alpha-haemolytic activity as determined by lysis of erythrocytes on 5% sheep blood agar. This is associated with the presence of the haemolysin gene (*hly*) (Murase et al., 2012).

The presumptive pathogenic *Escherichia coli* isolates were assessed for the expression of O157 antisera, resulting in a positive agglutination reaction for 12 of the isolates. This finding supports the identification of pathogenic *E. coli*; however, it is noteworthy that Medina et al. (2012) have documented instances of positive latex agglutination reactions occurring in non-O157 Shiga toxin-producing *E. coli* (STEC) strains. The presumptive pathogenic isolates were subsequently cultured on Eosin Methylene Blue (EMB) agar, where one of the positive isolates displayed a characteristic green metallic sheen. Nevertheless, the propensity for false positive results in subjective tests, such as those conducted using culture media, necessitated the consideration of additional colony morphologies, including purple and pink variants, for selection (Manafi and Kremsmaier, 2001). Furthermore, statistical analysis yielded no significant relationship between the isolation of *E. coli* and the subsequent isolation and characterisation of *E. coli* O157 on CT-SMAC agar.

These 34 isolates were identified based on their biochemical reaction presentation. It was clear that *E. coli* O157 was not the suspected isolate identity. These 34 isolates were from the family Enterobacteriaceae, which included predominantly *Enterobacter* spp and to a lesser degree *Serratia* spp, *Citrobacter* spp, and *Acinetobacter* spp. This is not uncommon as Barr et al. (1977) suggested limitations in the biochemical identification approach. The biochemical reactions observed, such as citrate utilisation and hydrolysis of urea, were consistent with expectations for Enterobacteriaceae that enable these isolates to survive in nutrient-depleted aquatic environments (Béchade et al., 2021). The common inability to ferment sorbitol confirms the observation made on the CT-SMAC agar, with isolate 28 (MR6_11) being the exception, possibly indicating the adaptive capabilities amongst Enterobacteriaceae in selective environments (Janda and Abbott, 2021). Common traits such as glucose fermentation, gas production, and hydrogen

sulphide production are known to be associated with Enterobacteriaceae genera (Murray et al., 2010).

The presence of indole production in strains nine and thirty-five indicates that these strains can convert tryptophan to indole, a biochemical reaction frequently observed in pathogenic and opportunistic Enterobacteriaceae (Salyers & Whitt, 2002). Indole production has been linked to biofilm formation and virulence, further underlining the potential pathogenicity of these isolates in aquatic environments (Boehm et al., 2020). The diverse biochemical profiles observed among strains isolated from different sites, such as the Setumo Dam and the Schoonspruit and Mooi River, highlight the influence of environmental factors on microbial metabolic expression. For instance, the Schoonspruit and Mooi River strains demonstrated a higher frequency of positive reactions across biochemical tests compared to Setumo Dam strains, suggesting that these environments may offer more favourable conditions for the expression of metabolic capabilities (Schultz et al., 2018). This variation in biochemical traits between water bodies could reflect differences in nutrient availability, pollution levels, or other ecological factors that influence microbial adaptability and metabolic flexibility (Li et al., 2019).

The enzymes haemolysin, β -galactosidase, catalase, and DNase were predominantly produced by the isolates, indicating their high expression among Enterobacteriaceae in aquatic environments. This finding is consistent with prior studies that emphasise the roles of these enzymes in bacterial survival and pathogenicity (Boehm et al., 2020; Perez et al., 2015). The presence of haemolysin-producing strains poses a public health risk, as these enzymes contribute to the invasiveness of pathogens and can exacerbate infections in humans and animals. Similarly, β -galactosidase was ubiquitously produced, suggesting that lactose metabolism may be a common trait among these isolates, potentially facilitating their survival in environments with varied nutrient sources, as supported by studies that link β -galactosidase activity to nutrient adaptability in Enterobacteriaceae (Wallenfels and Weil, 1972). However, this research detected the production ability of only one isolate for β -glucuronidase in the Mooi River, often associated with pathogenic *Escherichia coli* strains (Satoh et al., 2021; Dabek et al., 2008).

The production of catalase and DNase across nearly all isolates reinforces the hypothesis that these enzymes are critical for bacterial persistence in challenging environments. Catalase protects bacteria from oxidative stress, which is a common condition in surface waters exposed to sunlight and other environmental stressors (Watanabe et al., 2023). DNase production is also significant as it facilitates biofilm formation and immune evasion, both of which enhance bacterial survival and colonisation in diverse environments (Al-Wahaibi et al., 2019). The consistent presence of these enzymes highlights the potential for biofilm formation among these strains, raising concern about waterborne infections and the persistence of ARB in surface water systems.

The Setumo Dam isolates, for instance, showed high production rates of serine proteinase but did not produce β -glucuronidase or lecithinase, suggesting that environmental conditions at the Setumo Dam may selectively favour serine proteinase activity. This enzyme plays a role in bacterial invasion by degrading host proteins, indicating that Setumo Dam may harbour strains with pathogenic potential, particularly for human health. Enterobacteriaceae commonly reprogramme their metabolism to catabolise dietary L-serine, which enables their competitive fitness in the inflamed gut (Kitamoto et al., 2020, Pokharel et al., 2019).

The ubiquitous lipase production among Setumo Dam isolates suggests that lipid substrates are likely available in this environment, possibly due to organic matter from agricultural runoff, wastewater discharge, or other anthropogenic activities contributing to lipid-rich effluents, which is further exacerbated by rainfall (Gray et al., 2017). Such availability of lipid substrates may create a selective pressure favouring lipase-producing bacteria, enhancing their ability to thrive and persist in nutrient-limited or variable environments. This finding aligns with studies demonstrating that lipase activity in surface water bacteria is often associated with areas impacted by organic pollution, as these enzymes facilitate the breakdown of complex organic compounds, thus supporting microbial growth and metabolic diversity (Hachemi et al., 2017). The high prevalence of virulence factors among these Enterobacteriaceae isolates emphasises the urgent need to implement efficient water management strategies to reduce risks to public health emanating from pollution. Therefore, future research directions should include the long-term monitoring of such sources, and effective intervention strategies for water quality improvement in terms of anthropogenic impacts versus natural remediation processes must be considered.

ESBL enzymes, known for conferring resistance to β -lactam antibiotics, including penicillins and cephalosporins, are a significant public health concern as they limit treatment options for infections caused by Enterobacteriaceae (Paterson, 2006). This study observed the presence of ESBL-producing Enterobacteriaceae among the isolates, with 75% of strains from both the Setumo Dam and Mooi River and an overall prevalence of 62%, emphasising the significant risk posed by ARB in these surface water systems. This high prevalence of ESBL-producing strains is consistent with findings from previous studies indicating that water bodies near agricultural runoff, WWTPs, and urban settlements often serve as reservoirs for ARB (Blaak et al., 2015, Raseala et al., 2020). Wastewater effluents have been shown to contain antibiotic residues and resistant bacteria, creating hotspots for the development and spread of resistance in natural water bodies (Oliveira et al., 2023). The absence of ESBL production among isolates from the Schoonspruit River contrasts sharply with the other sites and suggests that local environmental factors, such as lower anthropogenic input or reduced antibiotic presence, may influence the selection pressure for ESBL production (Akpan et al., 2020).

The disk diffusion method revealed that all 34 isolates were susceptible to Gentamycin, Ciprofloxacin (97%), Chloramphenicol (91%) and Trimethoprim (74%). These patterns observed in surface water from the Mooi River, Schoonspruit River and Setumo Dam were consistent with similar reports in South Africa (Tsholo et al., 2024, Chukwu et al., 2019, Fadare and Okoh, 2021). This could be because gentamycin binds to the bacterial 30S ribosomal subunit, resulting in errors in protein synthesis that lead to bacterial death. The other reason for its effectiveness against Enterobacteriaceae may be due to its limited use and specific prescription habits used in clinical settings (Kadurugamuwa et al., 1993, Dillard et al., 2022). Furthermore, the three other classes that show susceptibility patterns to these Enterobacteriaceae could be due to target site modification or mutations as reflected in Table 2.1 in the literature review chapter (Chin et al., 2001, Kirmusaoğlu et al., 2019). Interestingly, the site downstream from the WWTP sampled along the Wonderfonteinspruit revealed that isolate 34 was resistant to all the eight classes of antibiotics tested, except for gentamycin and ciprofloxacin. Previous mention was made of the susceptibility of these compounds. However, from the same water sample, isolate 35 showed the least resistance to the classes of antibiotics tested with resistance to nalidixic acid and erythromycin. These two isolates are different genus types and it is possible that they originate from different pollution sources. Isolate 34 may have been exposed to higher levels of antibiotics in the environment (Tsholo et al., 2024, Pallecchi et al., 2007).

The intrinsic resistance of Enterobacteriaceae to erythromycin was confirmed in this study, and the results obtained are consistent with the literature (Iredell et al., 2016, Araújo et al., 2021). Clinicians do not prescribe erythromycin for infections where *E. coli*, *Klebsiella* spp, and *Enterobacter* spp are the causative agents (Iredell et al., 2016). According to the 2021 report from the NDH in South Africa (Surveillance for antimicrobial resistance and consumption of antimicrobials in South Africa) it was reported that sulphamethoxazole and extended-spectrum penicillins account for 13% and 28% respectively of total antibiotics used in 2020. The common use of these antibiotics is visible in the antibiotic phenotype of these isolates obtained during this study. Tsholo and colleagues also reported marked resistance to sulphamethoxazole and extended-spectrum antibiotics (Tsholo et al., 2024). This warrants a change in the prescription of antibiotics such as amoxicillin, cefazolin, ampicillin and sulphamethoxazole. Sulphamethoxazole is often prescribed in combination with trimethoprim in clinical settings. However, in agricultural settings where it is used as a growth-promoting and infection-preventing agent in animals, sulphamethoxazole is more commonly used than trimethoprim (Chang et al., 2019, Ronquillo and Hernandez, 2017, Brüssow, 2015). This is to confirm the patterns observed in this research, where the source of contamination is speculated to be as a result of agricultural activity.

Enterobacteriaceae infections are a major public health concern due to the rise in extended-spectrum β -lactam resistance (Nakai et al., 2016). Common infections caused by

Enterobacteriaceae include urinary tract infections (UTIs), bloodstream infections (sepsis), respiratory tract infections, and gastrointestinal infections that could result in diarrhoea, abdominal cramping and dehydration (Lim et al., 2019). These are life-threatening risks to human beings should they be infected with the strains isolated by this study. What further complicates the treatment is that many of these strains are ESBL-producing Enterobacteriaceae. These results emphasise the urgent need for continuous antimicrobial resistance surveillance and water quality management to mitigate the public health risks associated with antibiotic resistant Enterobacteriaceae in surface water systems of South Africa.

5.1.3 Genotypic identification and characteristics

Results obtained in this work contribute to the genotypic characterisation and determination of the antibiotic-resistant profile among Enterobacteriaceae isolated from surface water systems. These findings correspond with previous studies that echo microbial populations' ecological and public health importance. The result obtained in this study agrees with what was obtained from other works. The *Citrobacter* species is the most prevalent species, with 44% among the isolates. For example, the study of Singh et al. (2020) conducted in the Himalayan region reported the dominance of *Citrobacter* species in potable spring water. It stressed that it is an integral part of aquatic ecology. This is consistent across different locations, underpinning its adaptability and persistence in various water systems.

The profile of AMR that results from the current study is distressing and corroborates various other studies. The most alarming observation could be the high prevalence of the *Int1* gene in the isolates, which accounts for as high as 79%. According to Ahmed et al. (2021), *Int1* is a marker for anthropogenic pollution, indicating common environmental contamination. Besides this, other resistance genes found included *AmpC* (76%) and *sul2* (44%), which agrees with observations made in various studies where such resistance profiles were recorded in environmental samples from rivers. For instance, Kristiansson et al. (2011) reported high levels of *sul2* in antibiotic-contaminated river sediments, suggesting that effluent and sediment serve as reservoirs for these resistance genes. Similarly, Bueno et al. (2017) highlighted that *sul2* and other ARGs are commonly found in Gram-negative bacteria in natural environments, particularly near point sources of pollution such as WWTPs. This is corroborated by studies indicating that agricultural runoff and wastewater discharge significantly contribute to the dissemination of ARGs in aquatic ecosystems (Nappier et al., 2020).

The screening for virulence genes showed that all the isolates expressed the housekeeping gene *uidA*, while 50% of the isolates harboured the *hlyA* gene responsible for haemolysin production. Studies have documented that haemolysin production is not limited to clinical isolates but is also

prevalent among environmental strains of *E. coli*. For instance, Ibrahim et al. (2014) found that 40% of *E. coli* isolates from the Tigris River produced haemolysin, indicating a significant presence of this virulence factor in environmental samples. This finding aligns with the work of Konno et al. (2012), who reported the presence of haemolysin genes in various *E. coli* strains, including those derived from non-clinical sources, suggesting that these genes can be disseminated in the environment. The ability of environmental strains to produce haemolysin raises concerns about their potential to cause disease, particularly in vulnerable and immunocompromised populations (Moeinizadeh and Shaheli, 2021). The concern for public health increases when considering the ability of screened isolates due to the presence of the intimin gene (*eae*) that is responsible for bacterial adhesion and colonisation of host cells (Hasson et al., 2022; Ateba and Mbewe, 2014).

5.1.4 Whole genome sequencing

Surface water in the environment is frequently overlooked. This study initially misidentified four *Citrobacter* species as *E. coli* after conventional phenotypic microbiological tests. However, advanced taxonomic classification methods such as ANI and pangenome analysis clarified their identity as *Citrobacter* species. Traditional identification methods for these species are not recommended due to their frequent inaccuracies (Nayar et al., 2014). The findings revealed that environmental *Citrobacter* strains are distributed across various genetic clusters, raising concerns about the potential transmission of ARGs between humans, animals, and the environment. Research done on surface water in South Africa lacks sufficient knowledge on *Citrobacter* species' prevalence. Notably, only one genome of *C. murlinae* has been previously sequenced and reported, originating from a human faecal sample in Norwich, United Kingdom (Chen et al., 2019), making it the sole genome available in GenBank. While *C. portucalensis* has been well-characterised through various biochemical tests (Ribeiro et al., 2017), there is a lack of differential tests to distinguish between *Citrobacter* species.

5.1.4.1 Virulence genes present

In this study, the genomes of *C. braakii* can be differentiated from those of *C. murlinae* and *C. portucalensis* based on the genes responsible for the cell surface localisation of the CPS (*vexC*) and those involved in capsule polymer synthesis (*tvb* and *tvE*). Previous research has also indicated the presence of these genes (Yuan et al., 2019). The *C. murlinae* strain S24 could not produce catalase, which is corroborated by the lack of the *katB* gene. A gene cluster associated with the salmochelin siderophore system (*iroBCDEN*) was identified in *C. murlinae* S24, playing a significant role in the pathogenicity pathway that enhances colonisation. This gene cluster was initially described in *Salmonella enterica* (Baumler et al., 1998), and similar genes were

documented in various *E. coli* strains, indicating their acquisition through horizontal gene transfer (Sorsa et al., 2003). This is the first instance of the *iron-BCDEN* gene clusters reported in *Citrobacter* species, specifically within the *C. murlinae* genome. Additional virulence factors observed across all *Citrobacter* spp. strains were related to iron acquisition (*chu* and *ent*). To survive in a mammalian host during infection, pathogenic bacteria require iron, which they must secure (Carniel et al., 2001).

Notably, *C. murlinae* possesses an extra iron uptake gene, the *chuF* gene. This gene is recognised as an anearobiline reductase demonstrating kinetic cooperativity during the degradation biochemical process in *E. coli* O157:H7 (LaMattina et al., 2017). This iron gene cluster was identified among the *Citrobacter* spp., particularly in *C. koseri* strains (Yuan et al., 2019). However, this gene cluster was detected in all four sequenced genomes in the present study. The plasmids identified in this study are primarily linked to Type IV secretion systems. These Type IV secretion systems represent a widely distributed group that facilitates the secretion of macromolecules in both Gram-negative and Gram-positive bacteria and other eukaryotic cells (Grohmann et al., 2018). They are vital for bacterial interaction with their environments by secreting numerous virulence factors, especially in pathogenic bacteria (Blasey et al., 2022). Although the Type IV secretory genes are not fully characterised, they have been documented in most *Citrobacter* species genomes (Yuan et al., 2019).

5.1.4.2 Antibiotic resistance genes present

This research suggests that beta-lactamase genes (*bla*CMY) containing isolates are circulating in the river system. However, they are not limited to the environment, as some of these positive *bla*CMY genes containing isolates were isolated from plant and human isolates. The widespread use of broad-spectrum antibiotics might be the key factor in the emergence and dissemination of such elevated levels of antimicrobial-resistant strains in the freshwater of the Mooi River (Bosch et al., 2023). The present study illustrates the spectrum of *Citrobacter* in the freshwater of the Mooi River and indicates that *Citrobacter* is most often associated with MDR. This resistance might be due to the acquisition of resistance genes like β -lactamase, efflux pumps, or alternation in the porins, which act synergistically as a channel for drug entry to confer resistance among *Citrobacter* isolates (Yuan et al., 2019). The findings of *AmpC* gene presence gathered from PCR screening are confirmed by the WGS, which showed that these organisms are ESBL-producing Enterobacteriaceae. This was supported by the *Int1* gene, which indicated the anthropogenic pollution of antibiotics. Strains 21 and 25 were positively screened for *sul2* and *sul1* genes, respectively. Although these genes were not reported in WGS, this does not exclude their presence, as strict gene coverage percentages were applied for antibiotic screening.

The *qnrB13* gene, an indicator of fluoroquinolone resistance, was identified in the *C. portucalensis* strain S25 and compared with other genomes of *C. portucalensis* in this study. The chromosomal ARG *qnrB* has also been reported in other *Citrobacter* species (Ribeiro, 2015; Jacoby, 2011), indicating that *Citrobacter* species may be a source of chromosomal AR. This hypothesis is supported by species distribution, with more than 60% of occurrences found in *Citrobacter* species (Jacoby, 2011; Kehrenberg, 2008; Saga, 2013), as well as the prevalence of the *qnrB* gene in *C. freundii* strains isolated from human clinical samples (Park, 2007). Additional research affirms that the *Citrobacter* genus is a potential origin of the *qnrB* gene, as it is present in several species, including *C. freundii*, *C. braakii*, *C. youngae*, and *C. werkmanii*, albeit with allele variations specific to each species (Saga, 2013; Ribeiro, 2015). Moreover, analysis of the KEGG metabolic pathways further corroborated the resistance of these strains to fluoroquinolones, along with other virulent and antibiotic traits.

5.1.4.3 Metabolic pathways of genomic strains

The metabolic and phenotypic profiles of *Citrobacter* species, including *C. braakii*, *C. murlinae* and *C. portucalensis*, provide significant insight into their adaptability, virulence, and resistance mechanisms. The findings in this research align with trends observed in similar studies. The robust metabolic profile of *Citrobacter braakii* strain 21, characterised by active extracellular enzyme production and efficient iron-acquisition mechanisms, underscores its virulence and adaptability in host environments. Such traits have been linked to enhanced pathogenic potential and environmental fitness in related *Citrobacter* strains (Chen et al., 2019). Furthermore, its resistance to β -lactam antibiotics is supported by its genomic repertoire of β -lactamase enzymes, consistent with the findings of Samanta et al. (2017), who highlighted the prevalence of β -lactam resistance genes among *Citrobacter* species isolated from diverse environments. Similarly, *C. braakii* strain 23 exhibits comparable metabolic and resistance profiles. As demonstrated in this study, the ability to degrade extracellular lipids and proteins aligns with its environmental adaptability, which has been corroborated by observations in *Citrobacter* strains capable of utilising diverse nutrient sources (Hoque et al., 2020). Its reduced susceptibility to aminoglycosides further illustrates the selective advantage it gets from its resistance mechanisms.

Conversely, *C. murlinae* strain 24's ability to form biofilms and degrade polysaccharides highlights its potential for persistence in host environments. These traits are consistent with the findings of Wani et al. (2018), which emphasise biofilm formation as a critical factor in the pathogenicity of *Citrobacter*. The strain's resistance to macrolides also reflects the selective pressure exerted by antimicrobial use in its environment. Lastly, *C. portucalensis* strain 25 demonstrates metabolic versatility, correlating with its phenotypic ability to adapt to various

environmental conditions. Its extracellular enzyme production aligns with its invasive potential, as described in earlier studies on *Citrobacter* species (Zhang et al., 2021). Moreover, the observed resistance to β -lactams and tetracyclines is consistent with genomic analyses that reveal the prevalence of multidrug efflux systems and β -lactamase genes in *Citrobacter* (Olaitan et al., 2020).

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

This study provided a comprehensive analysis of the presence, distribution, and characteristics of Enterobacteriaceae in surface water systems, focusing on their public health implications and the environmental factors contributing to their prevalence. The findings reveal that antibiotic-resistant Enterobacteriaceae significantly contribute to water quality degradation and pose serious risks to human and ecosystem health. The isolation of pathogenic coliform bacteria, such as *Citrobacter* spp, *Enterobacter* spp, *Serratia* spp, *Escherichia coli*, *Hafnia* spp, *Klebsiella* spp and *Rahnella* spp is linked to faecal contamination and human activities, including agricultural runoff, stormwater runoff, wastewater discharge, urbanisation and informal settlements with improper sanitation infrastructure (Zhang et al., 2021; Khatri and Tyagi, 2015).

Water quality parameters, such as pH, temperature, and total dissolved solids (TDS), are crucial in microbial survival and adaptability. The high prevalence of faecal indicator organisms, particularly near WWTPs, highlights inefficiencies in waste management systems and the need for improved sanitation practices. Detecting MDR Enterobacteriaceae, particularly ESBL-producing strains, indicates a public health risk. In addition, standard treatment options currently in use, such as sulphamethoxazole, amoxicillin, cefazolin, and ampicillin, may no longer be effective in treating infections that could result from these strains. Moreover, these findings suggest the potential for gene transfer in these surface water systems, thus resulting in the spread of these antibiotic-resistant organisms to livestock, crops, and aquatic organisms in these freshwater sources.

The various characterisation tests showed diverse metabolic capabilities, the presence of virulence and ARGs, such as *Int1*, *AmpC*, *Sul1*, *sul2*, *blaTEM*, *blaCTX*, *blaOXA*, *blaNDM* and *blaKPC* genes. Further emphasising the nature of these strains and their abilities to express resistance to extended-spectrum β -lactamase and carbapenem treatment regimes. The WGS provided a comprehensive characterisation of the four selected *Citrobacter* spp. Worth mentioning is the presence of variants of the ESBL gene *blaCMY*. The Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathways suggested a strong interplay between environmental adaptability, metabolic flexibility, virulence and antibiotic characteristic confirmations. These findings emphasise the adaptive nature of these bacteria in nutrient-depleted environments and their potential to cause waterborne infections. According to Lim et al. (2019), the antibiotic-resistant Enterobacteriaceae could result in sepsis, UTIs, diarrhoea, gastrointestinal complications, and respiratory tract infections. This is further exacerbated by the

ESBL-producing ability, requiring more protracted and more expensive treatment regimes in an already burdened health sector (Pitout, 2010).

This research aimed to isolate, characterise and profile Enterobacteriaceae for antibiotic susceptibility and to detect ARGs using phenotypic and genotypic approaches. This aim was achieved by reaching the following objectives:

6.1.1 Quantify coliforms in samples from the selected surface waters

The quantification of total coliforms (TC) and *Escherichia coli* (*E. coli*) is essential for assessing water quality and detecting faecal contamination, as these indicators highlight potential health risks and the integrity of water systems. This study revealed significant contamination near WWTPs and areas with high anthropogenic activity, such as agricultural runoff and urban and informal settlements with inadequate sanitation. Seasonal factors such as rainfall and climate change could have contributed to high coliform contamination levels. Physical parameters (temperature, pH, and total dissolved solids) taken in situ in the surface water bodies promote the propagation of pathogens. This could include pathogenic strains like *E. coli* O157. As highlighted by the Green Drop report (DWS, 2022), inefficiencies in WWTP operations further contribute to microbial pollution of receiving surface water bodies. In addressing these issues, infrastructure enhancement, stricter compliance with water quality standards, and sustainable land use practices will be crucial to mitigate health risks and ensure the long-term sustainability of surface water resources, mainly since these surface water sources are used for full or partial reactional activities, agricultural activities, and potentially as drinking water sources for communities from informal settlements.

6.1.2 Isolation, characterisation and identification of pathogenic Enterobacteriaceae from among the coliforms and screen bacterial isolates recovered for extracellular and ESBL enzyme productivity

A total of 34 Enterobacteriaceae isolates were selected based on selective and differential screening using chromogenic culture media. These isolates were presumed to be the pathogen *E. coli* O157. Many of these culture-based approaches are subject to biases and false positives (Figure A.7), necessitating further biochemical assays. These isolates showed biochemical reactions characteristic of Enterobacteriaceae and a presumptive biochemical identification. However, the biochemical identification's reliability has been questioned by Popovic et al. (2022), which is consistent with the findings observed in this study. One cannot rely solely on biochemical identification. Thus, Sanger sequencing was used to supplement this. The production of extracellular enzymes such as haemolysin, catalase, DNase, and lipase demonstrates their adaptability to survive in nutrient-depleted conditions and a capacity for virulence and survival

under environmental stressors. These enzymes facilitate biofilm formation, immune evasion, and nutrient metabolism, which enhance bacterial persistence, pathogenicity, and host colonisation.

Particularly concerning is the high prevalence of ESBL-producing Enterobacteriaceae, observed in 75% of isolates from the Setumo Dam and Mooi River, highlighting a potential infection risk for the public. Furthermore, the findings suggest that anthropogenic activities, including agricultural runoff, wastewater discharge, urbanisation, and informal settlements, are critical drivers of virulence factors and AR in these bacterial populations. In contrast, the Schoonspruit River isolates did not grow on ESBL chromogenic media, which could be due to geographic locality variation (Coertze and Bezuidenhout, 2019; Saravanan et al., 2018). These results emphasise the need for targeted water management strategies and systematic monitoring to address the spread of virulence and resistance factors, protecting public health and aquatic ecosystems. The isolation and characterisation of ESBL-producing Enterobacteriaceae from the Setumo Dam and the Mooi River suggest that these water sources could serve as transmission routes for the spread of these priority pathogens as listed by the WHO (2019; see also Jesudason, 2024).

6.1.3 Screen bacterial isolates recovered from water samples for antibiotic susceptibility patterns, virulence, ESBL, carbapenems and antibiotic-resistant genes.

The antibiotic phenotypes, genotypes, and virulence factors of Enterobacteriaceae highlight their significant public health risks and adaptability in aquatic environments. While most isolates were susceptible to gentamycin, ciprofloxacin, chloramphenicol, and trimethoprim, resistance to widely used antibiotics such as sulphamethoxazole and extended-spectrum β -lactam antibiotics reflects the impact of agricultural and clinical antibiotic use. The prevalence of ESBL-producing strains and resistance genes like *Int1*, *AmpC*, and *sul2* point to the role of anthropogenic pollution in spreading antimicrobial resistance. Additionally, virulence factors such as the *hlyA* gene for haemolysin production and the *eae* gene for bacterial adhesion increase the pathogenic potential of these isolates, posing severe risks, particularly to vulnerable and immune-compromised populations. These findings stress the urgency of monitoring and managing AR and virulence in surface water systems to protect public health and guide effective water resource interventions.

6.1.4 Unravelling the whole genome of four *Citrobacter* species

The findings of this study highlight the critical role of AR, virulence genes, and metabolic pathways in characterising the pathogenic potential of *Citrobacter* species in surface water systems. High-resolution genomic analyses identified species such as *C. braakii*, *C. murlinae*, and *C.*

portucalensis, revealing significant genetic diversity and highlighting the limitations of traditional 16S rRNA gene identification methods. WGS data allows for a deep dive into the characteristics of Enterobacteriaceae, particularly the metabolic pathways that could speak to the association between AR and heavy metals (Singer et al., 2016). ARGs such as *bla*CMY and *qnr*B13 variants indicate the widespread dissemination of resistance traits, likely driven by anthropogenic pollution and the extensive use of broad-spectrum antibiotics in agricultural settings.

Virulence genes, such as those for iron uptake (*iroBCDEN* and *chuF*), capsule synthesis (*tviB* and *tviE*), and Type IV secretion systems, were prevalent, emphasising the capacity of these strains to establish infections and interact with host environments. Identifying metabolic pathways associated with resistance and virulence, such as those linked to fluoroquinolone resistance, Fosfomycin, and ESBL, shows these bacteria's adaptability and ecological fitness in aquatic ecosystems. Over and above the virulence and antibiotic indications, the metabolic pathway indicated that metal and AR can share structural and functional resistance systems (Singer et al., 2016; Bosch et al., 2023). Although metal resistance was not a specific focus for this study, these selected strains carry it. This is often associated with an ability to carry ARGs, as these genes could be found on plasmids (Pal et al., 2015; Di Cesare et al., 2016). This is consistent with the mining land use that affects these surface water systems.

6.2 Recommendations

The following recommendations are proposed to improve future research and address the shortfalls.

Firstly, with the advances in next generation sequencing such as shotgun analysis, microbial communities in surface water could be determined before following the culture-based methods and phenotypic characterisation approach. This would provide better insight into the types of bacteria in the surface water, ultimately enabling the selection of the best complementary culture-based approach. This would be like taking a CT scan of the environment so that scientists do not go in mindlessly to conduct research, but have a point of reference and a complementary approach to address what the CT scan revealed.

Although significant strides have been made with molecular techniques, the ability to culture a microbe will forever be the cornerstone of microbiology. Therefore, it is crucial to grow a pure culture in the laboratory and to preserve it for further downstream analysis or research studies. This was evident during the discovery of antibiotics. It is still relevant today due to the potential for simulating environments to study these organisms to determine their behaviour and characteristics, mimicking climate change variables, and for many other advances in science, medicine and industrial solutions.

WWTPs contribute to water sustainability by treating wastewater to a quality that allows for its reuse in irrigation, industrial processes, and even potable water supplies in advanced systems. This is especially important in South Africa, which is a water-stressed nation. Having adequate and working WWTP facilities is one of the ways to achieve SDG 6 set by the UN. However, many of these WWTPs have been identified as significant contributors to faecal contamination and the proliferation of ARB in aquatic ecosystems (Adomako et al., 2021; Suzuki et al., 2019). Strengthening the capacity of WWTPs to comply with microbial discharge standards and continuously monitoring effluents for faecal indicators and ARGs will help address these challenges effectively (Fadare & Okoh, 2021; Cohen et al., 2020).

Community education and public health initiatives should also be prioritised. Awareness campaigns on the importance of safe water use and hygiene practices can reduce the prevalence of waterborne diseases caused by Enterobacteriaceae (Malavi et al., 2018). Proper waste disposal practices, especially in areas with limited sanitation infrastructure, are essential to prevent the contamination of water bodies (Américo-Pinheiro et al., 2021). Additionally, fostering sustainable agricultural practices can significantly reduce nutrient runoff and antibiotic residues that promote the growth and persistence of Enterobacteriaceae in surface waters (Ronquillo & Hernandez, 2017). Reducing the use of antibiotics as growth promoters in livestock and ensuring proper waste management in farming areas are critical interventions (Brüssow, 2015; Chang et al., 2019).

Although the one-health approach encourages all role players to come together to address the AR apocalypse predicted for 2050, there is an urgent need for health practitioners, farmers, veterinary practitioners, scientists and regulatory authorities to share information. This may include conferences to bridge the gap across disciplines and to encourage relationships across sectors. Another approach could be injecting funds to foster collaboration across sectors to address the rising antibiotic crisis. Collaboration among government agencies, research institutions, and community organisations is essential for implementing effective water safety plans that protect human health and ecosystems (Malavi et al., 2021; Singh et al., 2020).

Policy and regulatory frameworks should be reinforced to establish and enforce stricter water quality standards, particularly in regions with high contamination risks. Comprehensive surveillance programmes should be implemented to track ARB in water systems and evaluate the success of mitigation efforts (Kostoglou, 2023; Salamandane et al., 2021). Furthermore, investment in research and technological advancements is crucial for developing cost-effective and efficient methods for detecting faecal contamination and resistance genes in water. Innovative remediation approaches, such as biofilters and constructed wetlands, could be explored to enhance water purification processes (Fuentes-Rivas et al., 2020).

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ANNEXURE

Table A.1: Target water quality ranges reported by the Department of Water and Sanitation for recreational and agricultural use of water

Parameter	Safe Target Water Quality Range (TWQR)			
	Full-contact recreation	Intermediate contact recreation	Agricultural Irrigation	use: Agricultural use: Livestock
Temperature (°C)	No Data	No Data	No Data	No Data
pH (pH units)	6.5 – 8.4	No Data	6.5 – 8.4	No Data
Total dissolved solids (mg/L)	No Data	No Data	≤260	0 - 1000
Total Coliforms (counts/100)	0 - 130	0 - 1000	No Data	0 - 1000
<i>Escherichia coli</i> (counts/100)	0 - 130	No Data	1 - 1000	No Data

Table A.2: The physical and microbiological water quality analysis of the Mooi River, Schoonspruit River and Setumo Dam.

River system	Date	Sampling site	Label	Temp (°C)	pH	TDS (mg/L)	Total Coliforms (MPN/CFU* per 100 ml)	<i>E. coli</i> (MPN/CFU* per 100 ml)
Mooi River	Oct-17	Wonderfonteinspruit downstream of WWTP	WF1	21.70	8.02	644.00	2419.60	2419.60
Mooi River	Oct-17	Wonderfonteinspruit upstream of WWTP	WF2	21.50	8.04	655.00	2419.60	547.50
Mooi River	Oct-17	Klerkskraal	MR1	21.50	8.50	357.00	1553.10	325.50
Mooi River	Oct-17	Muiskraal	MR2	23.20	8.54	327.00	2419.60	179.30
Mooi River	Oct-17	Boskop A	MR3	21.50	8.13	482.00	2419.60	248.10
Mooi River	Oct-17	Boskop B	MR4	22.50	8.49	459.00	2419.60	816.40
Mooi River	Oct-17	Thabo Mbeki Bridge	MR5	20.80	8.47	460.00	2419.60	2419.60
Mooi River	Oct-17	Trimpark	MR6	22.30	7.85	655.00	2419.60	2419.60
Mooi River	Oct-17	Pedestrian Bridge	MR7	25.60	8.04	472.00	2419.60	1119.90
Mooi River	Oct-17	Viljoenroad Bridge	MR8	24.00	8.20	523.00	2419.60	770.10
Average±STDEV				22.46±1.45	8.23±0.25	503.40±117.36	2332.95±274.01	1126.56±936.02
Schoonspruit River	Nov-17	Ventersdorp raw water	SCH1	20.30	8.63	375.00	165.00	58.30
Schoonspruit River	Nov-17	Bodenstein	SCH2	18.60	8.24	627.00	2419.60	161.50
Schoonspruit River	Nov-17	Klerksdorp Dam	SCH4	22.50	9.24	354.00	2419.60	66.30
Schoonspruit River	Nov-17	Voortrekker	SCH5	20.70	8.04	604.00	1732.90	1732.90
Schoonspruit River	Nov-17	Downstream WWTP	SCH6	22.30	8.28	846.00	2419.60	2419.60
Schoonspruit River	Nov-17	Orkney	SCH7	22.30	8.64	817.00	2419.60	344.80
Average±STDEV				21.12±1.54	8.51±0.43	603.83±209.48	1929.38±906.96	797.23±1019.48
Setumo Dam	May-17	Point Closest to the Wastewater	S1	24.7	7.67	2197.00	300.00*	300.00*
Setumo Dam	May-17	Distribution site 1	S2	22.9	8.54	791.00	300.00*	300.00*
Setumo Dam	May-17	Distribution site 2	S3	23.6	8.84	758.00	300.00*	300.00*
Setumo Dam	May-17	Point closest to Drinking-water Facility	S4	23.3	9.13	748.00	75.00*	73.00*
Average±STDEV				23.63±0.77	8.55±0.63	1123.50±715.90	243.75±112.50	243.25±113.50

Table A.3: Antibiotic susceptibility raw data for the 34 selected isolates

Source	Sample name	Sample no.	K	N	CN	S	TE	E	CIP	AML	KZ	AMP	C	W	NA	RL
SD	S2_R1.2	5	22	24	24	20	18	9	30	8	NCZ	10	25	23	25	NCZ
SD	S3_R1.1	6	20	22	25	20	20	10	40	NCZ	NCZ	NCZ	30	25	27	NCZ
SD	S1_R1.1	7	20	20	20	15	16	10	35	NCZ	NCZ	NCZ	20	20	22	NCZ
SD	S4_R1.1_2	8	22	20	22	12	17	8	25	NCZ	NCZ	NCZ	25	25	22	NCZ
SSR	SCH6_27	9	20	20	20	12	18	NCZ	24	NCZ	NCZ	NCZ	20	14	NCZ	NCZ
SSR	SCH6_29	10	10	16	20	NCZ	NCZ	NCZ	20	NCZ	NCZ	18	20	NCZ	18	NCZ
SSR	SCH2.1_21	11	18	16	16	11	15	12	35	NCZ	NCZ	NCZ	18	25	21	NCZ
SSR	SCH6_31	12	12	20	18	10	NCZ	NCZ	22	10	NCZ	14	22	NCZ	17	NCZ
SSR	SCH2.1_19	13	17	18	20	8	16	NCZ	28	NCZ	NCZ	NCZ	22	21	18	NCZ
SSR	SCH6_31_2	38	30	25	30	20	20	25	35	22	30	30	25	NCZ	30	25
MR	MR7_4	14	20	22	20	10	16	NCZ	38	NCZ	NCZ	13	23	26	20	NCZ
MR	MR6_12	15	15	17	20	9	13	12	30	NCZ	NCZ	13	20	26	20	NCZ
MR	MR6_27	16	15	15	20	NCZ	NCZ	NCZ	32	NCZ	NCZ	NCZ	20	NCZ	18	NCZ
MR	MR6_9	17	17	22	25	8	NCZ	NCZ	26	16	18	18	23	NCZ	18	NCZ
MR	MR6_8	18	16	16	18	11	14	10	28	NCZ	NCZ	NCZ	22	16	16	NCZ
MR	MR6_33	19	20	18	20	10	15	NCZ	32	NCZ	NCZ	NCZ	20	20	12	NCZ
MR	MR6_19	20	20	20	20	NCZ	15	NCZ	30	NCZ	NCZ	11	20	22	18	NCZ
MR	MR6_1	21	16	16	16	NCZ	14	NCZ	22	NCZ	NCZ	NCZ	20	20	22	NCZ
MR	MR6_6	22	16	15	16	14	16	8	24	NCZ	NCZ	NCZ	17	21	20	NCZ
MR	MR6_48	23	12	12	15	11	16	NCZ	30	8	NCZ	10	18	21	16	NCZ
MR	MR6_8_2	24	22	20	22	16	15	8	30	15	18	15	22	23	20	NCZ
MR	MR6_22	25	16	20	20	12	15	NCZ	26	12	NCZ	11	18	20	13	NCZ
MR	MR6_32_2	31	20	18	18	13	NCZ	NCZ	22	NCZ	NCZ	8	21	12	18	NCZ
MR	MR6_3	26	18	16	18	12	12	NCZ	30	NCZ	NCZ	NCZ	22	18	20	NCZ
MR	MR6_11	28	16	20	17	11	13	16	30	NCZ	NCZ	NCZ	20	18	22	NCZ
MR	MR6_16	29	15	16	17	9	15	NCZ	25	7	NCZ	NCZ	17	16	15	NCZ
MR	MR6_10	30	20	22	20	16	16	NCZ	30	NCZ	NCZ	NCZ	20	20	21	NCZ

Table A.3 continues...

Source	Sample name	Sample no.	K	N	CN	S	TE	E	CIP	AML	KZ	AMP	C	W	NA	RL
MR	MR6_32	37	12	12	12	8	15	NCZ	25	NCZ	NCZ	NCZ	20	20	22	NCZ
MR	MR6_20	32	18	20	16	12	15	NCZ	25	NCZ	NCZ	NCZ	18	18	22	NCZ
MR	MR6_15	33	16	16	16	12	13	NCZ	25	NCZ	NCZ	NCZ	20	20	20	NCZ
MR	MR6_21	36	16	20	17	15	14	NCZ	25	NCZ	NCZ	NCZ	22	18	21	NCZ
MR	WF1_18	34	16	16	16	NCZ	NCZ	NCZ	21	NCZ	NCZ	NCZ	12	NCZ	NCZ	NCZ
MR	WF1_42	27	16	15	16	NCZ	NCZ	NCZ	25	NCZ	NCZ	NCZ	18	NCZ	16	NCZ
MR	WF1_5	35	16	16	16	12	13	NCZ	25	NCZ	NCZ	10	20	20	20	NCZ

Red=Resistant; Black=Intermediate; Green=Susceptible; SD: Setumo Dam; SSR: Schoonspruit River; MR: Mooi River. Antibiotic disks: K: Kanamycin; N: Neomycin; CN: Gentamycin; S: Streptomycin; TE: Tetracycline; E: Erythromycin; CIP: Ciprofloxacin; AML: Amoxicillin; KZ: Cefazolin; AMP: Ampicillin; C: Chloramphenicol; W: Trimethoprim; NA: Nalidixic acid; RL: Sulfamethoxazole. NCZ: No Clearance Zone

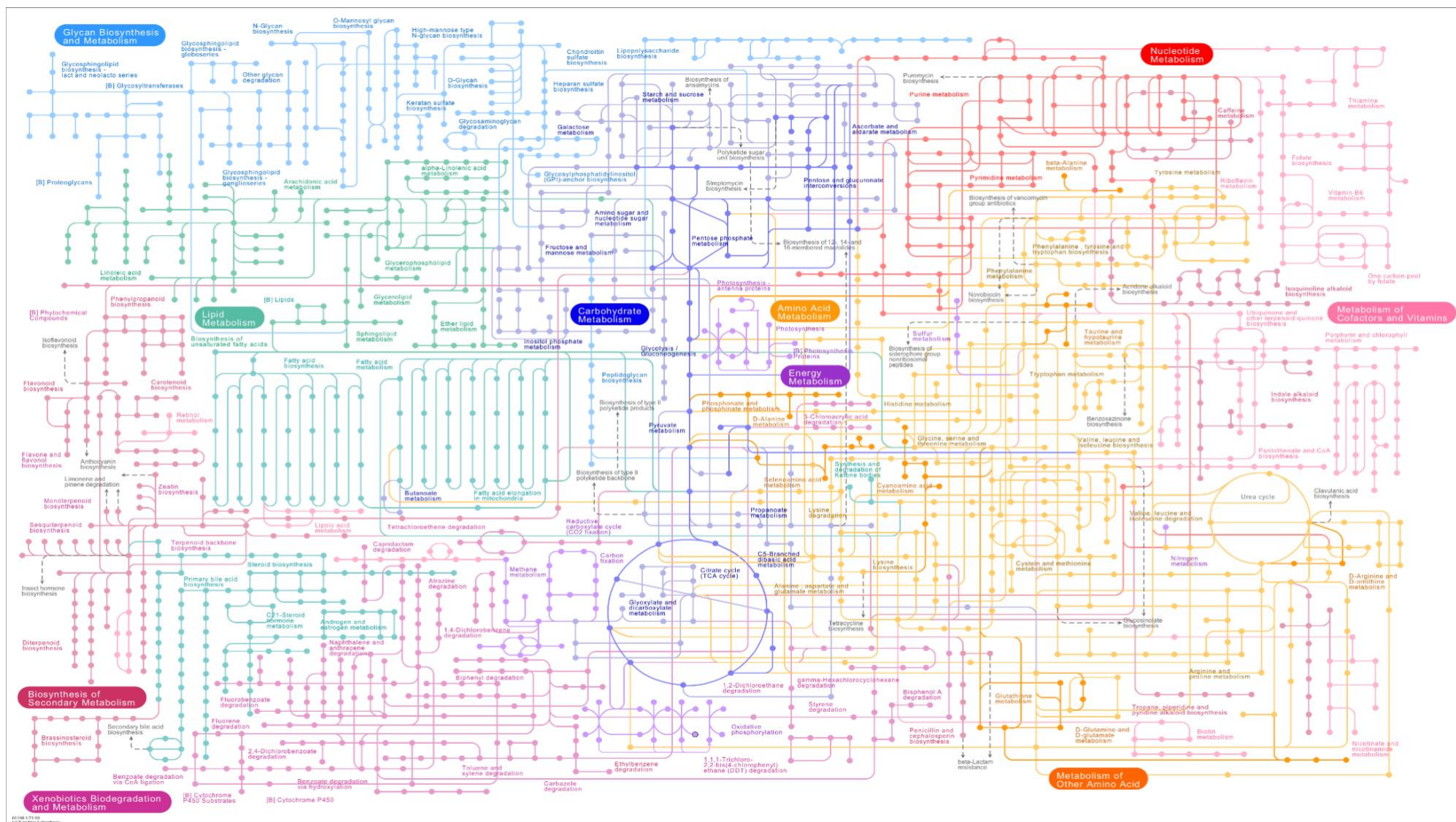


Figure A.1: The Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathway of *Citrobacter braakii* Strain 21.

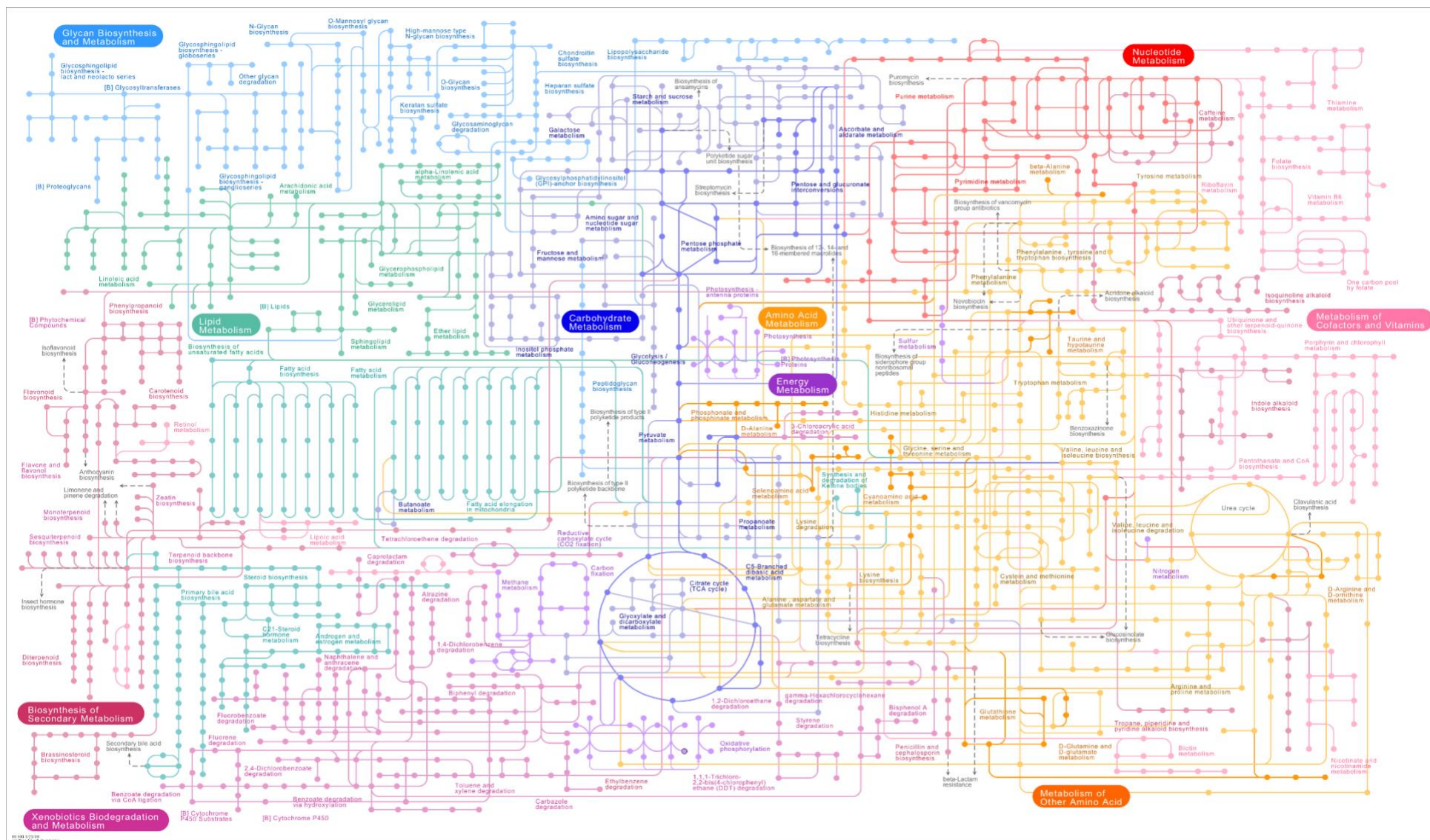


Figure A.3: Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathway of *Citrobacter murlinae* Strain 24.

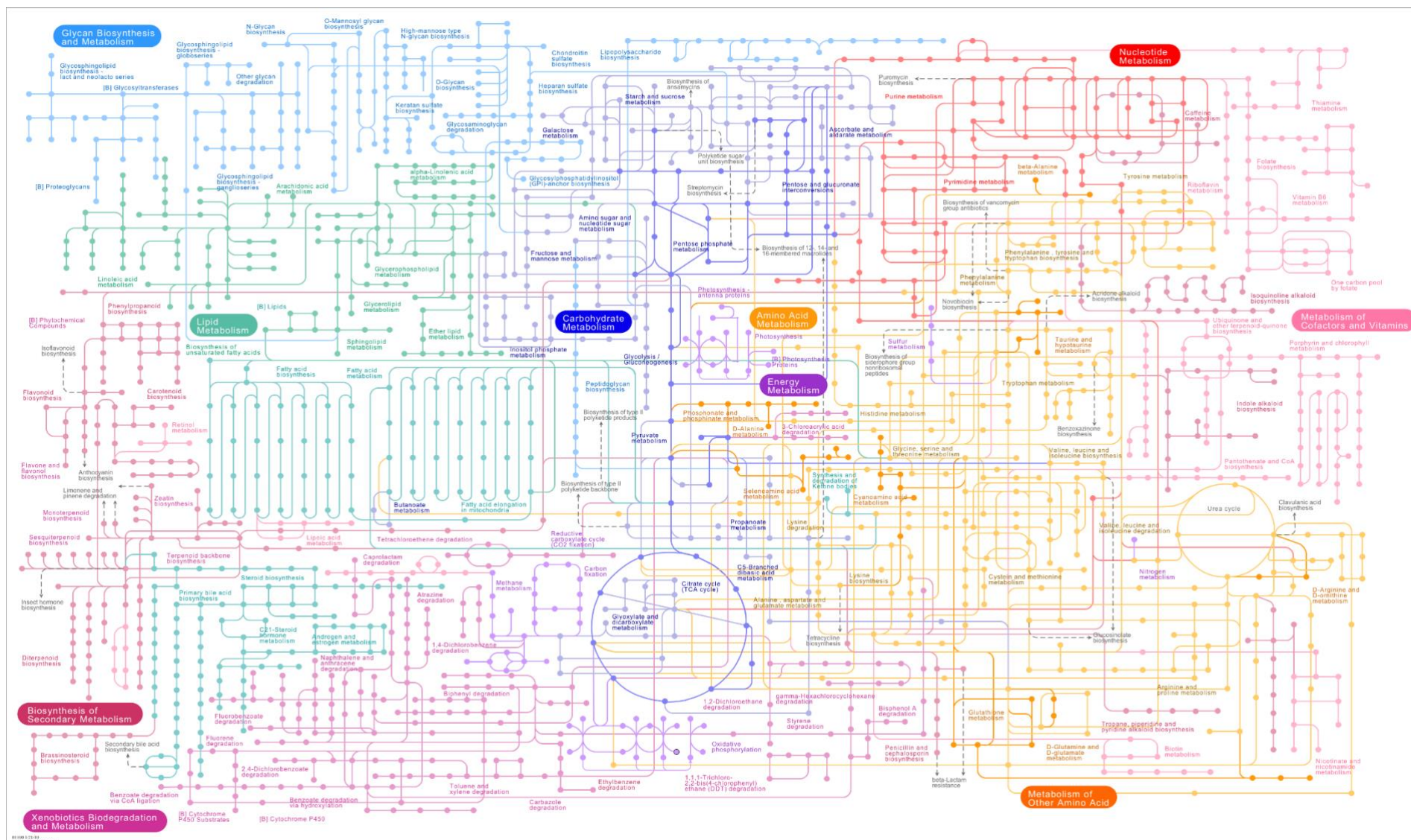


Figure A.4: Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathway of *Citrobacter portucalensis* Strain 25.

Table A.4: Comparison of three methodologies to identify Enterobacteriaceae to genus and or species level

Strain	Sample Name	Biochemical ID	Sanger Sequence ID	Whole Genome Sequence ID
5	S2_R1.2	Unidentified	<i>Serratia fonticola</i>	ND
6	S3_R1.1	Unidentified	<i>Escherichia coli</i>	ND
7	S1_R1.1	<i>Serratia marcescens</i>	<i>Citrobacter freundii</i>	<i>Serratia fonticola</i>
8	S4_R1.1_2	<i>Serratia marcescens</i>	<i>Serratia fonticola</i>	ND
9	Sch6_27	Unidentified	<i>Citrobacter freundii</i>	ND
10	Sch6_29	Unidentified	<i>Enterobacter cloacae</i>	ND
11	Sch2.1_21	Unidentified	<i>Citrobacter freundii</i>	ND
12	Sch6_31	Unidentified	<i>Citrobacter freundii</i>	ND
13	Sch2.1_19	Unidentified	<i>Citrobacter spp.</i>	ND
38	Sch6_31_2	<i>Acinetobacter iwoffii</i>	<i>Rahnella aquatilis</i>	ND
14	MR7_4	<i>Enterobacter gergoviae</i>	<i>Citrobacter portucalensis</i>	ND
15	MR6_12	<i>Enterobacter cloacae</i>	<i>Citrobacter spp.</i>	ND
16	MR6_27	<i>Enterobacter gergoviae</i>	<i>Klebsiella spp.</i>	ND
17	MR6_9	<i>Enterobacter gergoviae</i>	<i>Hafnia alvei</i>	<i>Enterobacter hormaechei</i>
18	MR6_8	Unidentified	<i>Citrobacter portucalensis</i>	<i>Enterobacter ludwigii</i>
19	MR6_33	<i>Enterobacter cloacae</i>	<i>Klebsiella variicola</i>	ND
20	MR6_19	<i>Enterobacter cloacae</i>	<i>Enterobacter spp.</i>	ND
21	MR6_1	Unidentified	<i>Enterobacter spp.</i>	<i>Citrobacter braakii</i>
22	MR6_6	Unidentified	<i>Citrobacter freundii</i>	ND
23	MR6_48	<i>Enterobacter cloacae</i>	<i>Citrobacter portucalensis</i>	<i>Citrobacter braakii</i>
24	MR6_8_2	Unidentified	<i>Citrobacter portucalensis</i>	<i>Citrobacter murlinae</i>
25	MR6_22	<i>Citrobacter freundii</i>	<i>Citrobacter freundii</i>	<i>Citrobacter portucalensis</i>
26	MR6_3	Unidentified	<i>Hafnia alvei</i>	<i>Hafnia paralvei</i>
28	MR6_11	Unidentified	<i>Citrobacter portucalensis</i>	<i>Enterobacter hormaechei</i>
29	MR6_16	<i>Enterobacter gergoviae</i>	<i>Citrobacter spp.</i>	<i>Hafnia paralvei</i>
30	MR6_10	<i>Enterobacter gergoviae</i>	<i>Rahnella aquatilis</i>	ND
37	MR6_32	<i>Enterobacter gergoviae</i>	<i>Enterobacter chuandaensis</i>	ND
32	MR6_20	Unidentified	<i>Rahnella aquatilis</i>	ND
33	MR6_15	<i>Enterobacter gergoviae</i>	<i>Rahnella aquatilis</i>	<i>Hafnia paralvei</i>
36	MR6_21	<i>Serratia marcescens</i>	<i>Rahnella spp.</i>	ND
31	MR6_32_2	Unidentified	<i>Rahnella aquatilis</i>	ND
35	WF1_5	Unidentified	<i>Escherichia coli</i>	<i>Escherichia coli (ETEC)</i>
27	WF1_42	Unidentified	<i>Citrobacter koseri</i>	<i>Hafnia paralvei</i>
34	WF1_18	<i>Enterobacter cloacae</i>	<i>Citrobacter portucalensis</i>	<i>Providencia rettgeri</i>

Table A.5: Virulence genes of *Citrobacter* spp. encoding adhesins, environmental stress response, iron uptake, siderophores, biofilm formation and antigens determined in this study

Virulence response	Virulence genes	Product and mechanism
Environmental stress response Iron uptake	<i>cheA, cheD, cheW, cheY</i> <i>chuA, chuS, chuT, chuU, chuW, chuX, chuY</i>	Chemotaxis proteins production Heme-binding proteins
Biofilm formation	<i>csgA, csgB, csgC, csgD, csgE, csgF, csgG</i>	Curli production assembly
Iron uptake	<i>entA, entB, entC, entE, entF, entS</i>	Multi-enzyme complex formation carries out a series of biochemical reactions in the bacterial cell.
Siderophores	<i>fepA, fepB, fepC, fepD, fepG</i>	Ferrienterobactin transporter/ binding protein
Adhesins	<i>fimC, fimD, fimF, fimH</i>	Chaperone and usher proteins associated with virulent adhesion.
Adhesins	<i>flgG, flgH</i>	Flagellar protein associated with virulent adhesion.
Adhesins	<i>fliA, fliC, fliG, flil, fliM, fliN, fliP</i>	Flagellar motor switch proteins associated with virulent adhesion.
Salmochelinsiderophores	<i>iroB, iroC, iroD, iroE, iroN, shuA</i>	iron-salmochelins utilization plays a role in pathogenicity.
Biofilm formation	<i>misL</i>	Autotransporter protein
Environmental stress	<i>ompA</i>	Outer membrane protein A
Pathogenesis	<i>katB</i>	Resistance against peroxidase and facilitate phagocyte-mediated killing.
Antigens	<i>tviB, tviC, tviD, tviE</i>	Polysaccharide biosynthesis protein involved in capsule polymer synthesis.
Antigens/Biofilm Formation	<i>vexA, vexB, vexC, vexD, vexE</i>	Polysaccharide export protein produced that encodes for cell surface localization of the Capsular Polysaccharides.

Table A.6: The antibiotics resistance genes detected using Comprehensive Antibiotic Resistance Database. The genes and their associated drugclass, treatments and resistance mechanism of actions are indicated

Gene	Drugclass	Antibiotic treatment	Resistance mechanism
<i>acrAB/ToIC</i>	Multidrug/ macrolides	Global health threat	Efflux pump
<i>acrD</i>	Aminoglycoside	Severe infections of the abdomen, urinary tract and the heart	Efflux pump
<i>acrEF/ToIC</i>	Multidrug	Global health threat	Efflux pump
<i>bacA</i>	Bacitracin	Preventative for skin infections	Efflux pump
<i>CMY-59</i>	β -lactams (ESBLs)	Pulmonary, urinary, skin and soft tissue infection	Antibiotic inactivation
<i>CMY-74</i>	β -lactams (ESBLs)	Pulmonary, urinary, skin and soft tissue infection	Antibiotic inactivation
<i>CMY-77</i>	β -lactams (ESBLs)	Pulmonary, urinary, skin and soft tissue infection	Antibiotic inactivation
<i>CMY-83</i>	β -lactams (ESBLs)	Pulmonary, urinary, skin and soft tissue infection	Antibiotic inactivation
<i>emrAB/ToIC</i>	Quinolones	Community and severe hospital acquired infections	Efflux pump or mutations
<i>emrD</i>	Multidrug	Global health threat	Efflux pump
<i>mdfA</i>	Multidrug	Global health threat	Efflux pump
<i>mdtABC/ToIC</i>	Aminocoumarin	Lyme disease	Efflux pump
<i>mdtE*F</i>	Multidrug	Global health threat	Efflux pump
<i>mdtG</i>	Phosphonic acid/Macrolides	Urinary tract infections	Efflux pump
<i>mdtH</i>	Quinolone	Septicemia, urinary, joint, bone, tissue and pulmonary infections	Efflux pump
<i>mdtK</i>	Quinolone	Septicemia, urinary, joint, bone, tissue and pulmonary infections	Efflux pump
<i>mdtM</i>	Phenicol	Bacterial conjunctivitis (eye infection) and Cholera treatment	Efflux pump
<i>msbA</i>	Nitroimidazole	Parasitic, anearobic bacterial infections and prevent post-operative infections	Efflux pump
<i>patA</i>	Quinolone	Septicemia, urinary, joint, bone, tissue and pulmonary infections	Efflux pump
<i>pmrEFC</i>	Peptide antibiotic	Multidrug resistant infections	Antibiotic target alteration
<i>qnrB13**</i>	Quinolone	Septicemia, urinary, joint, bone, tissue and pulmonary infections	Efflux pump
<i>yojI</i>	Peptide antibiotic	Multidrug resistant infections	Efflux pump

** only determined in *C. portuculansis* strain S25



Figure A.5: Sampling Images

Water sampling was performed following the EPA-described grab sample. Physical parameters were taken onsite and recorded at each site. While at the site, JCJ Fourie captured these images. The site labelled b (Schoonspruit River) is of interest with visible organic matter, consistent with findings of faecal pollution at this site. The site labelled d was taken from the Setumo Dam sampling, showing the informal settlement around the water source.

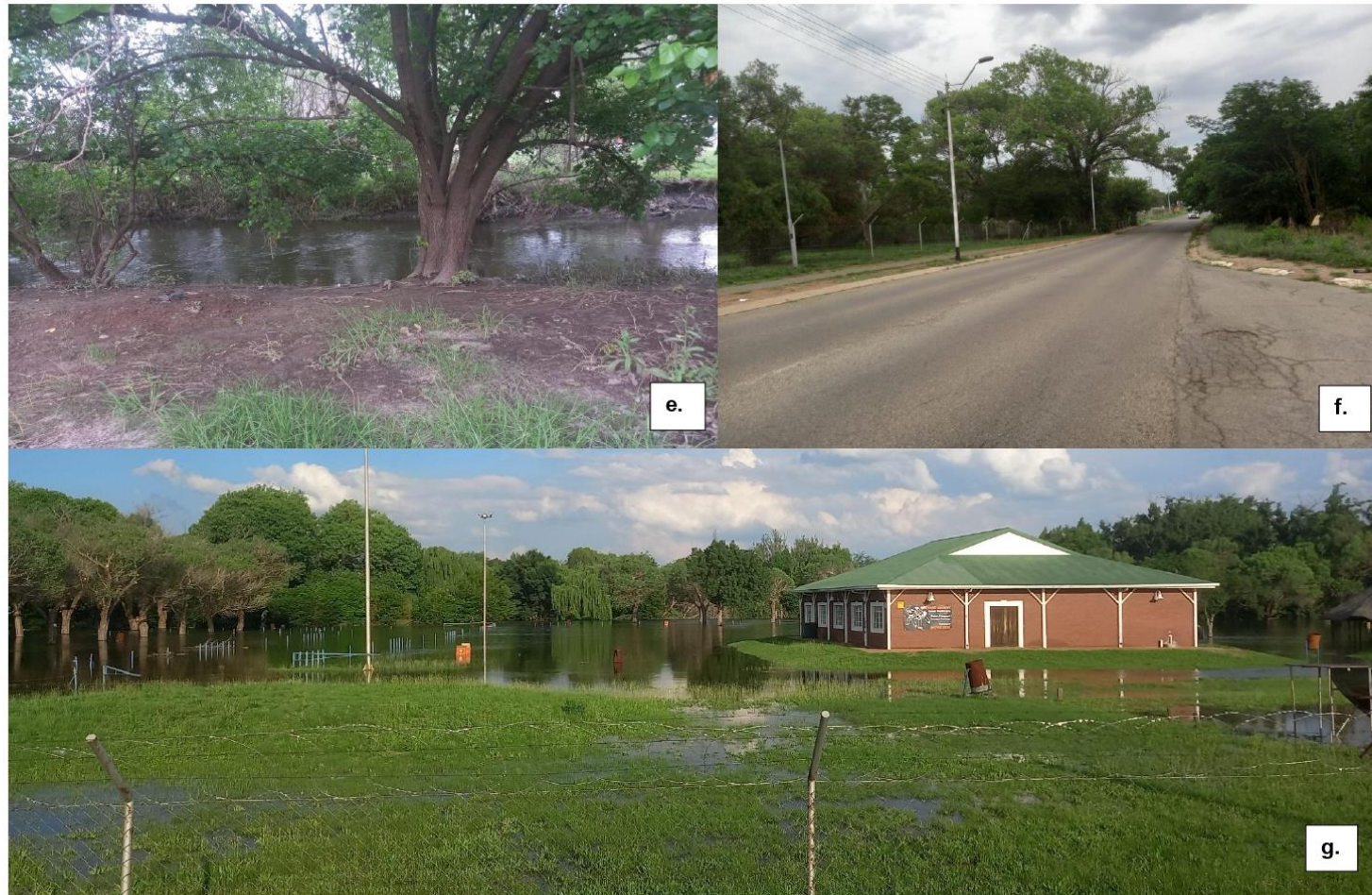


Figure A.6: Recreational site along the Mooi River

These images are from site MR6 from the Mooi River, a popular recreational site. However, in 2022, Potchefstroom was among the many towns in South Africa that were experiencing floods, and this was the same for this site. The four ESBL-producing *Citrobacter* species that underwent WGS were from this site, and flooding could have resulted in these organisms' spread.

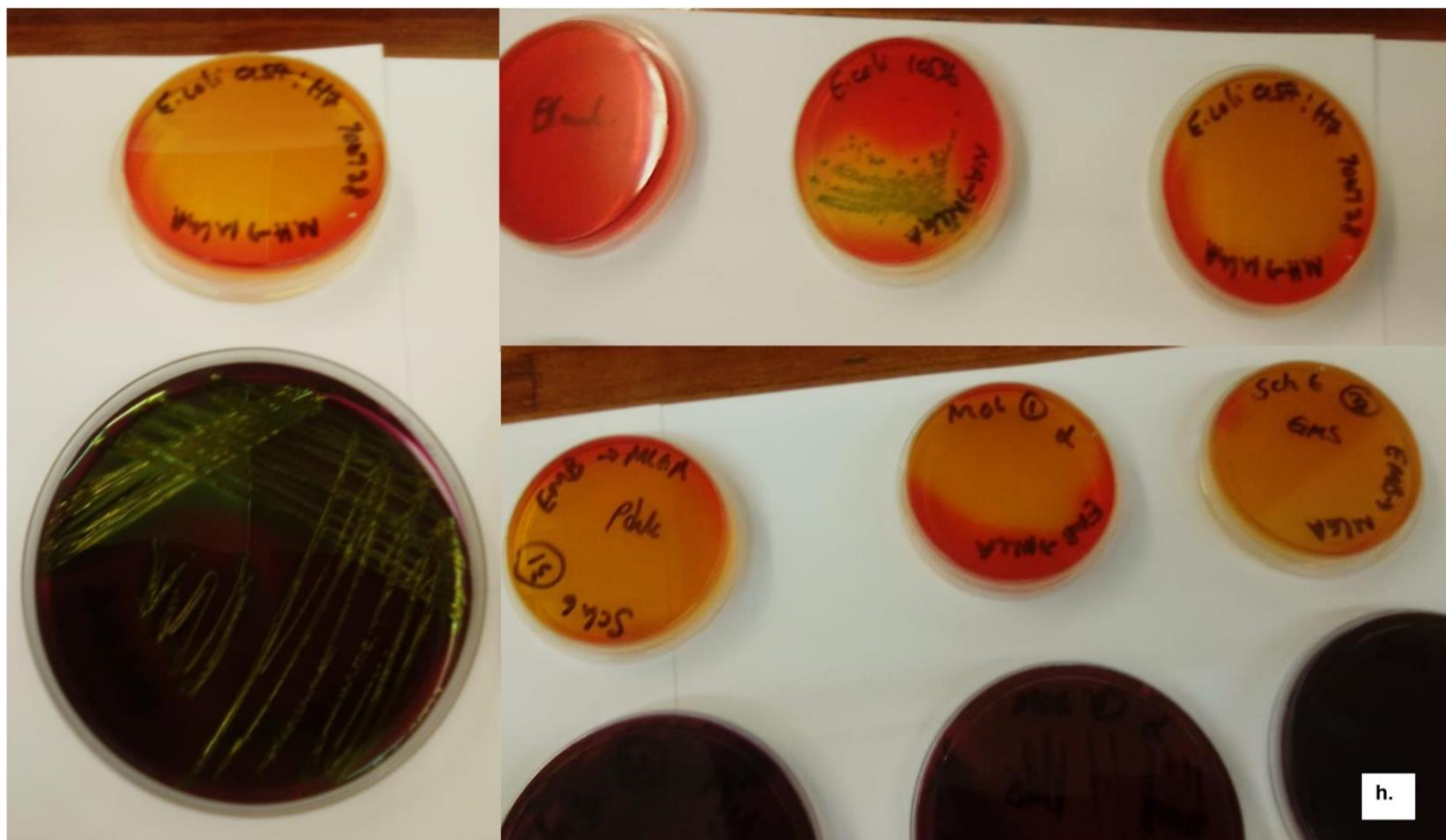


Figure A.7: *E. coli* O157:H7 unique appearance on MLG agar

MLG agar and EMB agar are common agars used to culture coliforms; however, our finds reveal that *E. coli* O157:H7 ATCC 700728 does not produce the green pigmentation on the MLG agar. Expected growth of the *E. coli* O157:H7 ATCC 700728 culture was observed on EMB agar in a green metallic sheen appearance. Furthermore, the presumptive *E. coli* O157 was consistent with the presentation of *E. coli* O157:H7 ATCC 700728. Another ATCC strain of *E. coli* was used as a positive control on MLG agar, this being *E. coli* ATCC 10536 and produced the expected green colony pigmentation.

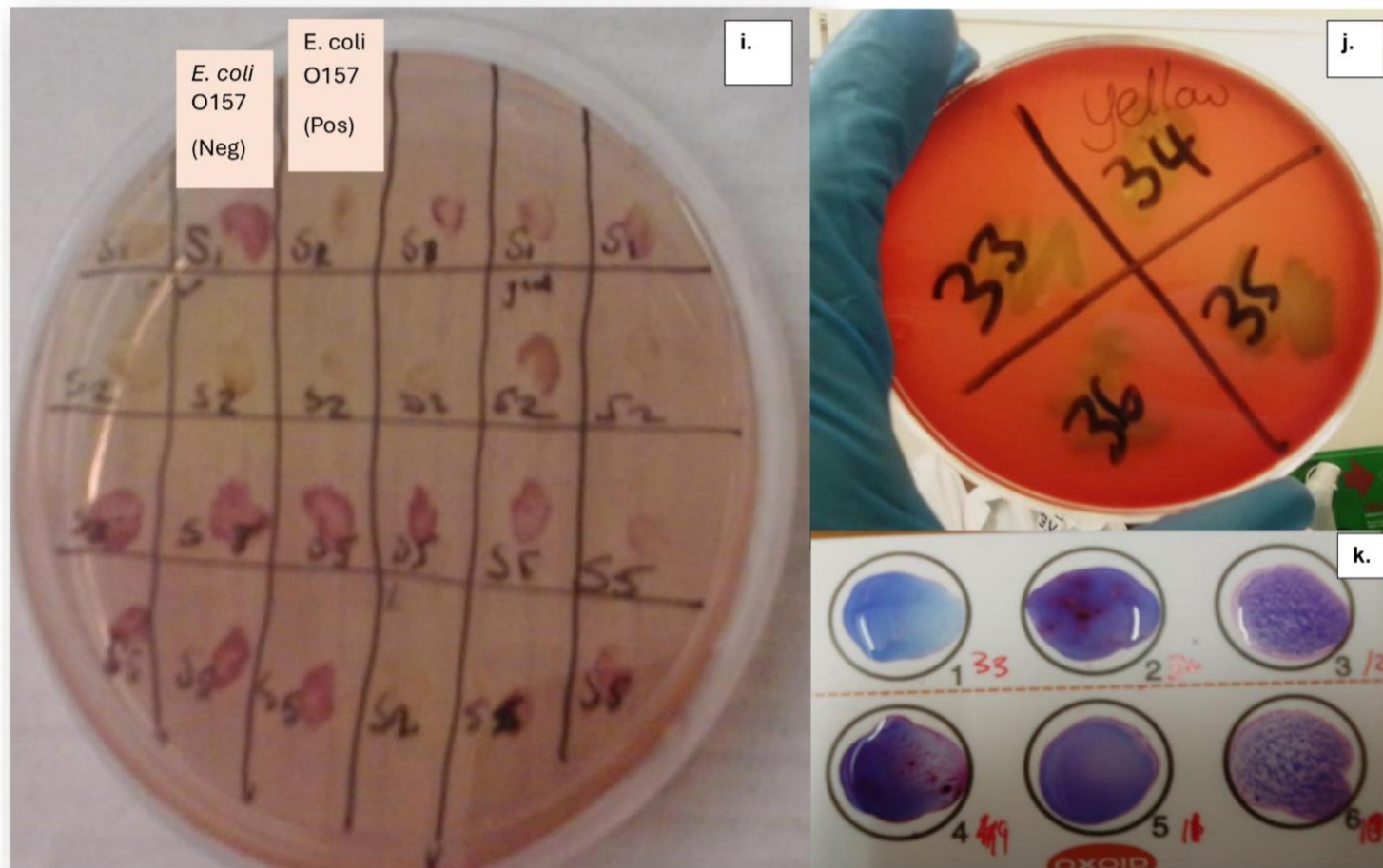


Figure A.8: Characteristic features of Enterobacteriaceae isolates

The CT-SMAC agar was crucial in deciding which colonies would be used downstream. The focus was selecting the clear (*E. coli* O157 positive) colonies for pathogenic testing on 5% Sheep Blood agar. The latter confirmed these organisms as potentially pathogenic, presenting as α -haemolysis as seen in block j. These presumptive pathogenic *E. coli* O157 isolates were serologically screened for agglutination ability with *E. coli* O157 antisera. Block k isolate 12 was latex positive, whereas isolate 33 tested negative.



Figure A.9: Extended-spectrum β -lactamase chromogenic agar

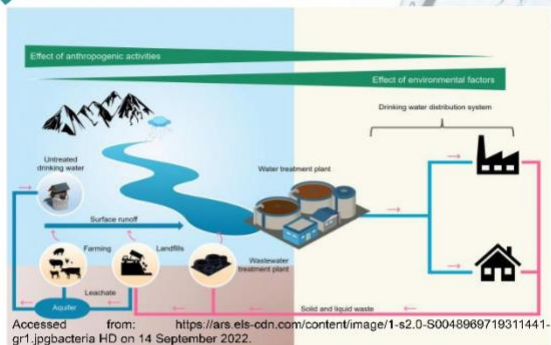
It was important to establish whether these pathogenic strains could produce extended-spectrum β -lactamase. The ESBL Chromogenic media aided in establishing this, and the results are reflected above. Colonies that appeared pink/red were likely ESBL-producing *E. coli* and the blue pigmented growth would likely be ESBL-producing *Citrobacter*, *Enterobacter* and *Klebsiella* species.

Multidrug Resistant *Enterobacteriaceae* from Surface Water in the North-west Province, South Africa

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1 Introduction



- In developing countries, high mortality rates are predicted that would be attributed to AMR, reaching 4.5 million by 2050.
- Surface waters (dams, springs and rivers) are potential reservoirs and carriers of antibiotic resistant bacteria and genes .

2 Methods



- Surface waters are used in agriculture for irrigation and watering of animals as well as raw water for drinking water. This could thus affect animals, humans and plant health negatively (One-Health Concept).

3 Results

Table 1: Multi-Drug Resistant *Enterobacteriaceae* Profiles

Source	Representative No. of Isolates	Genus Identification	Antibiotic Resistance Profile	
			Antibiogram Profile	Antibiotic Resistance Gene Profile
Control Strains	1	<i>Escherichia coli</i> ATCC 10536	Strep	<i>Int1</i>
	1	<i>Escherichia coli</i> O157 ATCC 700728	Sulpha	<i>Int1-ampC-sul1-sul2-blaTEM</i>
Setumo Dam	4	<i>Serratia fonticola</i>	Sulpha-Amp-Cepha-Amox	<i>Int1-ampC-sul2-blaTEM-blaCTX</i>
Schoonspruit River	5	<i>Citrobacter freundii</i>	Strep-Sulpha-Amp-Cepha-Amox-Nal	<i>Int1-ampC-sul1-sul2-blaOXA</i>
Mool River	9	<i>Citrobacter freundii</i>	Strep-Sulpha-Amp-Cepha-Amox-Nal-Chlor	<i>Int1-ampC-sul1-sul2</i>
	1	<i>Enterobacter chuandaesis</i>	Strep-Sulpha-Amp-Cepha-Amox	<i>ampC-sul1-blaTEM</i>
	2	<i>Escherichia coli</i>	Strep-Sulpha-Nal	<i>Int1-ampC-sul1</i>
	9	<i>Hafnia paralvei</i>	Strep-Sulpha-Amp-Cepha-Amox	<i>Int1-ampC-sul1-sul2-blaTEM-blaNDM</i>

Strep: Streptomycin; Sulpha: Sulphamethoxazole; Amp: Ampicillin; Cepha: Cephazolin; Amox: Amoxicillin; Nal: Nalidixic acid; Chlor: Chloramphenicol

4 Conclusion

- Multidrug *Enterobacteriaceae* were isolated from selected surface waters from the North West Province of South Africa.
- Common antibiogram profiles included resistance to Sulpha-Amp-Cepha-Amox-Strep.
- Various antibiotic resistance genes were detected from the total genomic DNA of the species and associated with antibiograms of the representatives.
- Beta-lactamase ARGs were common. In all cases the *Int 1* gene were amplified.



Figure A.10: Poster presented at EDAR 2022.

RESEARCH

Open Access



The phylogenomic landscape of extended-spectrum β -lactamase producing *Citrobacter* species isolated from surface water

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Abstract

Background *Citrobacter* species are Gram-negative opportunistic pathogens commonly reported in nosocomial-acquired infections. This study characterised four *Citrobacter* species that were isolated from surface water in the North West Province, South Africa.

Results Phenotypic antimicrobial susceptibility profiles of the isolates demonstrated their ability to produce the extended-spectrum β -lactamase (ESBL). Whole genomes were sequenced to profile antibiotic resistance and virulence genes, as well as mobile genetic elements. *In silico* taxonomic identification was conducted by using multi-locus sequence typing and average nucleotide identity. A pangenome was used to determine the phylogenomic landscape of the *Citrobacter* species by using 109 publicly available genomes. The strains S21 and S23 were identified as *C. braakii*, while strains S24 and S25 were *C. murlinae* and *C. portucalensis*, respectively. Comparative genomics and sequenced genomes of the ESBL-producing isolates consisted of $n=91$; 83% *Citrobacter* species in which *bla*_{C_{MY-101}} ($n=19$; 32,2%) and *bla*_{C_{MY-59}} ($n=12$; 38,7%) were prevalent in *C. braakii*, and *C. portucalensis* strains, respectively. Macrolide (*acrAB*-TolC, and *mdtG*) and aminoglycoside (*acrD*) efflux pumps genes were identified in the four sequenced *Citrobacter* spp. isolates. The quinolone resistance gene, *qnrB13*, was exclusive to the *C. portucalensis* S25 strain. *In silico* analysis detected plasmid replicon types IncHI1A, IncP, and Col(VCM04) in *C. murlinae* S24 and *C. portucalensis* S25, respectively. These potentially facilitate the T4SS secretion system in *Citrobacter* species. In this study, the *C. braakii* genomes could be distinguished from *C. murlinae* and *C. portucalensis* on the basis of gene encoding for cell surface localisation of the CPS (*vexC*) and identification of genes involved in capsule polymer synthesis (*tvbB* and *tvbE*). A cluster for the salmochelin siderophore system (*iro-BCDEN*) was found in *C. murlinae* S24. This is important when it comes to the pathogenicity pathway that confers an advantage in colonisation.

Conclusions The emerging and genomic landscapes of these ESBL-producing *Citrobacter* species are of significant concern due to their dissemination potential in freshwater systems. The presence of these ESBL and multidrug-resistant (MDR) pathogens in aquatic environments is of One Health importance, since they potentially impact the clinical domain, that is, in terms of human health and the agricultural domain, that is, in terms of animal health and food production as well as the environmental domain.

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Figure A.11: Research article published with data generated from this project

