A metagenomic snapshot of selected wastewater treatment plants in Vhembe Region, Limpopo, South Africa:

Investigating the resistome

by

Damien Jacobs

(17017009)

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in the Department of Biochemistry and Microbiology

Faculty of Science, Engineering and Agriculture

University of Venda

Thohoyandou, Limpopo, South Africa

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Abstract

Background: Water is crucial for human life. Rural communities often rely on natural water sources which may become polluted by wastewater due to various activities such as domestic use and agriculture. Antibiotic resistance genes (ARGs) may be transferred from wastewater to the environment and pose a global challenge they affect both human and animal-related sectors. Studying antibiotic resistance in wastewater treatment plants within Vhembe offers a representation of antibiotic resistance genes from entire communities. Knowledge of antibiotic resistance circulating in Vhembe has been sparsely studied. Metagenomics approaches allow for a broad overview of the resistome and the bacterial communities within environmental samples. **Aim:** To perform wastewater surveillance of antibiotic resistance genes and associated bacteria within Vhembe, Limpopo, South Africa, using a metagenomics approach. **Method:** A total of 32 sample duplicates were collected from the influents (n=18) and the effluents (n = 14) from nine wastewater treatment plants (WWTPs) around the Vhembe region, Limpopo, South Africa. One hundred milliliter was filtered using sterile cotton gauze and Wattman filter paper to remove debris and membrane filtered through 0.22um membrane filters to capture the bacteria within each sample. DNA was extracted directly from the 0.22 μm filters using a DNA miniprep kit. DNA was quantified using a spectrophotometer. Shotgun metagenomic sequencing was performed on DNA extracts. Open-source bioinformatics pipelines were used to process and analyze raw sequence data, uncovering information of the bacterial community composition and associated ARGs in wastewater. **Results:** Site observations reveal animal and human activities within and near the sites. ARG analysis revealed an overall number 0f 220 ARGs detected across the WWTPs. Thirty-six genes were common to influent samples and 16 in within effluent samples, encoding predominantly against macrolides, sulfonamides and tetracyclines, beta-lactamases, and aminoglycosides. Some unique ARGS were detected at sites near South African borders. Bacterial Diversity showed the predominance of some genera, such as *Arcobacter*, *Aeromonas*, *Pseudomonas* and *Acinetobacter*. Pathogens were predominantly enteric and pulmonary, with some being linked to animals in past studies. A notable increase in some members of *Mycobactericeae,* among other bacteria, was noted in effluents.

Keywords: Wastewater-surveillance, antibiotic-resistance, Metagenomics, Environmental, Water, Vhembe, Resistome

Declaration of Independent work

I, Damien Jacobs (Student number 17017009) declare that this research dissertation is my original work and has not been submitted for any degree at any other University or Institution. The work does not contain any other person's writing unless acknowledged and referenced accordingly.

Signed…………………………………….. **Date**…23-02-2024……..

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List of abbreviations

AR : Antibiotic resistance

ARG : Antibiotic resistance gene

DEWATS : Decentralized wastewater treatment systems

LC-MS : Liquid chromatography-mass spectrophotometry

MGE : Mobile Genetic elements’

PCR : Polymerase Chain Reaction

rRNA : Ribosomal RNA

WHO : World Health Organisation

WW : Wastewater

WWTP : Wastewater treatment plant

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Chapter 1: General introduction

1.1 Background

Water is an important resource for life, and by extension, human activities such as agriculture, industry and domestic use. Human activities associated with water have been observed to directly affect the quality of natural water sources in rural communities within the Vhembe District in the Limpopo Province of South Africa (Traore *et al.,* 2016). This has introduced a wide range of different pathogens such as bacteria, protists and viruses which cause gastroenteritis (Potgieter *et al.,* 2020). According to the Environment Protection Authority Victoria, wastewater (WW) is categorized depending on its origin: greywater from washing and bathing, blackwater stems from toilet waste, sewage which combines trade waste, blackwater and greywater and industrial wastewater from various businesses and industry (excluding sewage) (EPAV, 2022). Wastewater treatment involves multibarrier approaches with a combination of unit processes (chemical/biological treatments), unit operations (physical treatment) and water distribution have been essential in mitigating the impact of WW on the environment (Metcalf and Eddy 2003; Spellman, 2020). Human activities involving water generate WW which gets transported to WWTPs, localizing community-wide wastewater contaminants. If improperly treated, WW introduces a broad host of pollutants such as antibiotics and a wide array of pathogenic microorganisms into natural water (Osunmakinde *et al*., 2021, Sabri *et al*., 2020a; Oharisi *et al*., 2023). Antibiotic resistance occurs when a bacterium survives exposure to an antibiotic and gains the ability to prevent its uptake or metabolize it into harmless metabolites. According to the World Health Organization (WHO), a growing global challenge is antibiotic resistance, which threatens a country's public health and economy. Many sectors from healthcare, industry, and the public are negatively impacted by resistant bacteria from, humans, animals and wastewaters. Treatment options are becoming more challenging as antibiotic resistance increases (WHO 2015; WHO 2020). Several factors have been identified in Africa, including South Africa, to contribute to AMR, such as, poor adherence to national antibiotic prescription guidelines and excessive use of antibiotics in livestock (Abia *et al*., 2023).

Wastewater in South Africa has been found to contain multidrug resistant bacteria such as *E. coli, Enterococcus* and *Pseudomonas* from both influent sewage and final effluents in different settings (rural, urban, and peri-urban). The dissemination of antibiotic resistant bacteria (ARB) and their genes may thus allow widespread acquisition of antibiotic resistance in receiving water bodies, which may end up in animals and humans (Mbanga *et al*., 2021; Odjadjare *et al* 2012). Focusing on the relationship between the health of the environment, animals and people is termed the One Health approach and can be applied to investigate antibiotic resistance and pathogens present in a multifaceted way (Purohit *et al.,* 2017; Gholizadeh *et al.,* 2023). In rural areas of the Vhembe Region, Limpopo, South Africa, humans, and animals largely depend on natural water bodies such as rivers and dams, which are polluted by wastewater such as domestic sewage containing pathogenic bacteria like *E. coli* (Traore *et al*., 2016; Potgieter *et al*., 2020). Conventional methods such as polymerase chain reaction (PCR) and culturing are commonly used within the country due to their cost-effectiveness and sensitivity; however, information on antibiotic resistance in the environment is sparce in the Limpopo Province and may require broader approaches such as metagenomics (Abia *et al.,* 2023). Metagenomics involves the identification of all nucleotide sequences found within a microbial community in each sample and has been used to identify bacteria, antibiotic resistance genes (ARGs) and the bacteriophages that may transfer ARGs (Subirats *et al.,* 2016; Zhang *et al.,* 2023). This study provides an epidemiological “snapshot” of the bacteria and ARGs in selected WWTPs across the Vhembe District using shotgun metagenomics. This snapshot could increase the understanding of the ARGs circulating in the community and those being introduced into the aquatic environment.

1.2 Rationale

Increasing water scarcity within African countries, including South Africa, is exasperated by the pollution of natural water sources due to partially treated wastewater. Water scarcity is becoming increasingly challenging to combat due to factors such as population growth (Nansubuga *et al*., 2016). Rural areas within Vhembe are at risk of waterborne disease, potable water loss from various human activities, sewage pollution, inadequate wastewater treatment infrastructure and lack of proper sanitation within schools and clinics. Additionally, factors that may contribute to the spread of such disease may be a lack of knowledge within communities of pathogen transmission routes (Sibiya *et al*., 2013; Traore *et al*., 2016; Potgieter *et al*., 2020; Potgieter *et al*., 2021)

Wastewater, if properly treated, may provide a valuable resource attributed to the nutrients it contains and may alleviate the impact of water scarcity (Nansubuga *et al*., 2016). However, the green drop report shows that most WWTPs in the Vhembe District are in critical condition (DWS, 2023). This may lead to the escape of pathogenic microbes and ARGs that may further the burden of diseases that already circulate within water sources in Vhembe (i.e. gastroenteritis) (Potgieter *et al*., 2020). Some communities in Vhembe have been practicing wastewater reuse for activities such as agriculture, further risking their exposure to bacterial pathogens (Gumbo *et al.*, 2010).

Studies conducted in Vhembe on sewage-impacted water and the households that surround them have given key clues on the presence of bacteria such as *E. coli, V. cholerae* and *Salmonella* (Potgieter *et al*., 2020; Rakalaru *et al.*, 2023), However studying clinical samples from a community may be time-consuming and individuals harboring ARB may be missed in the wider population. There is a growing body of studies on antimicrobial resistance that have been conducted in Africa and South Africa; however, studies in Limpopo are scarce. This lack of knowledge adds to the issue of dealing with antibiotic resistance in the province. Detection and characterization methods such as PCR and Kirby disc diffusion, respectively are cost-effective and can establish vital phenotypic expression of antibiotic resistance (Abia *et al*., 2023).

Within Vhembe, studies on ARGs in the environment are scarce, additionally, wastewater treatment is not fundamentally geared towards ARG and antibiotic removal. Metagenomics can be applied to bridge our gap in understanding the circulating antibiotic resistance within the region due to its broad detection capabilities. “Catch-all” approaches such as metagenomics are pivotal in understanding the relationship between AMR, pathogens, humans, and animals within the environment due to their interconnectedness. To gain a full scope of the potential AMR and circulating bacterial pathogens within the region, wastewater may be a useful tool in combination with the broad detection capabilities offered by shotgun metagenomic sequencing.

1.3 Objectives:

1.3.1 Primary Objective

To perform a once-off wastewater survey of antibiotic resistance genes and associated bacteria within Vhembe, Limpopo, South Africa, using a metagenomics approach.

1.3.2 Secondary objectives

- To isolate DNA from wastewater samples and subsequently sequence the metagenome using next-generation sequencing (NGS).
- To identify the diversity of ARGs within the WWTPs of the Vhembe district
- To Identify the diversity of microbial taxa within the collected wastewater samples from the metagenomic data

1.4 Research questions:

- What are the predominating ARGs circulating within the communities?
- What are the predominating ARGs escaping into the environment through the effluents?
- What are the predominating pathogens circulating in selected WWTPs within Vhembe?

1.5 Hypothesis:

Antibiotic resistance genes and microbial diversity will be more prevalent in receiving influents than effluent wastewater within the Vhembe region.

Chapter 2: Literature review

2.1 Water, wastewater and antibiotic resistance

2.1.1 Antibiotic resistance gene dissemination in wastewater

Wastewater treatment plants serve as hotspots for ARG dissemination. These genes are from various sources where antibiotics are present or being overused, such as farms, abattoirs, and hospitals (Tanih *et al.,* 2015; Abia *et al*., 2023). In South Africa, both pathogenic bacteria and ARGs have been detected in environmental, animal, and clinical sources (Tanih *et al.,* 2015. Traore *et al.,* 2016). Antibiotics in low concentration would allow the development of antibiotic resistance within the environment. A study conducted in the Netherlands demonstrated that some antibiotics, such as sulfonamides, can persist through wastewater treatment and along rivers. ARGs have also been found to increase after wastewater treatment and downstream in receiving rivers (Sabri *et al*., 2020a). ARG presence, according to the study, also shows some seasonal variation; however, the seasonality may depend on the ARG in question or the water quality parameters, such as nitrites and dissolved oxygen, in addition to the seasonality of the Bacteria they aim to treat. Sewage sludge, the solid phase of wastewater, was also shown in that study to serve as an important reservoir for ARGs. Total suspended solids (TSS) removal is influential on the removal of antibiotics as the sorption of ARB to suspended particles may aid in their dissemination (Sabri *et al*., 2020a). Human activities were shown to influence the concentration of ARGs and antibiotics along rivers to their estuaries. Photodegradation was also observed to play a role in ARG survival and concentration in the river environment through the production of more stable metabolites. One ARG, *sul1*, encoding for sulfonamide resistance, maintained a constant concentration up to 20 km downstream from the WWTP (Sabri *et al.,* 2020b). Effluent quality parameters could thus be adjusted perhaps to include antibiotics and/or ARG removal in wastewater due to their persistence.

2.1.2 Mechanisms of wastewater treatment.

For WWTPs to be effective, a multi-barrier approach is necessary. This involves various WW treatment steps (Figure 2.1): pre-treatment, primary treatment, secondary treatment, and tertiary treatment. Secondary treatment of WW is commonly done using biological treatment such as activated sludge. Activated sludge releases suspended solids and has been shown to act as a reservoir ARGs antibiotics and bacteria due to their adsorption to the solid phase, and thus, their removal is important. Due to the nature of ARGs and their transference through different means, such as phages and mobile genetic elements (MGEs), it would make sense that processes which use bacteria would naturally accumulate ARGs over time. The type of secondary treatment, presence of additional treatments and factors like hydraulic retention time have been shown to influence the removal and persistence of ARGs (Lamba and Ahmmad., 2017; Sabri *et al.,* 2020a). For example, a study by Lamba and Ahmmad demonstrated that modified trickling filters and UV/Ozonation fared better in removing ARBs and ARGs than conventional activated sludge and anaerobic flow-through reactors. Chlorination was also investigated and may be a poor choice in removing ARGs due to its lower oxidation strength. The mechanisms involved in the tertiary treatment process are outlined in Table 2.1. It is thus important to consider which tertiary treatment is being used at a given treatment plant when considering surveillance and future plant improvements.

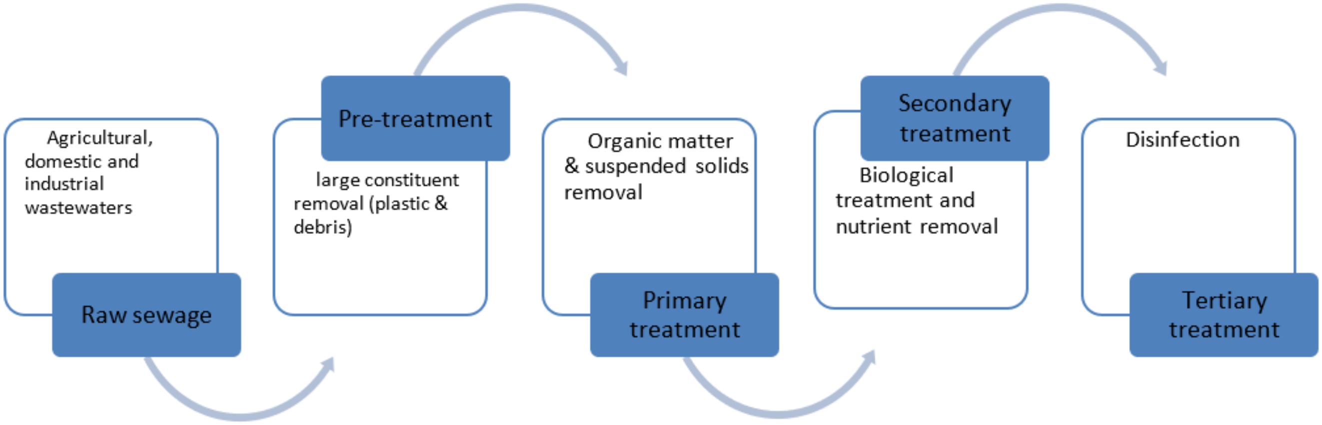


Figure 2.1: Flow chart of the various stages of wastewater treatment

Table 2.1: Outline of different disinfection methods

Method of disinfection	Factors influencing efficacy	Mechanisms of action	Advantages	Disadvantages	Citation
Chlorination	Exposure time. Concentration (%) used for disinfection. Influent water pH. Turbidity. Presence of ammonia.	Elemental chlorine (Cl) contacts water, hydrolysation occurs, forming hypochlorous acid (HOCl) and hypochlorite $^{-}$OCl), which are strong oxidative agents. Reduction of available oxygen and nutrients. Oxidation of available amino acids.	Economical, oxidation of metals also occurs, simultaneously clarifies waters via oxidation.	Formation of toxic by-products, polluting receiving bodies.	(Naidoo and Olaniran, 2014), (Singh *et al.,* 2021)
Ultraviolet Light	Effluent quality. UV light intensity. UV wave path length from lamp to target microbe.	UV emission by a mercury lamp. Deterioration of genetic material (200-300nm). Production of reactive oxygen species (hydroxyl radical). Dimerization of pyrimidine in DNA/RNA.	No need for handling or transporting potentially harmful chemical disinfectants. Reduced production of toxic pollutants and by-products.	Microbes tend to repair their DNA at low dosages of UV light. More effective in conjunction with other disinfection methods.	Naidoo and Olaniran, 2014), (Singh *et al.,* 2021)
Ozonation	Type of effluent being treated. Dependency on a stable power source.	Passage of oxygen through a strong electric field generates ozone: Oxidation of viral capsid proteins by generation of Protein hydroxides and protein hydroperoxides, Ozone reacts with tryptophan, methionine, cysteine, linoleic	Less environmental impact than other conventional means. Effective against various viruses Ozone may be generated onsite from the air.	Viruses are more tolerant to ozone than bacteria; indicator bacteria may not be sufficient for indicating viral removal. High operation costs.	(Naidoo and Olaniran, 2014), (Singh *et al.,*. 2021)
Hydrogen peroxide	Concentration (%).	H_2O_2 forms reactive –OH radicals damaging genetic material, proteins, and lipids.	No risk due to the decomposition of H_2O_2 to water and O_2 from UV light exposure. Effective at low doses (3% concentration is typically used).	Impractical for large-scale operations.	(Naidoo and Olaniran,2014), (Singh *et al.,* 2021)

			Effective against a wide range of microorganisms.		

2.1.3 Wastewater treatment and antibiotics/ARGs

Antibiotics can enter WWTPs from various human and animal sources, including hospitals and abattoirs (Abia *et al*., 2023). Effective wastewater treatment must address three essential pollutants: chemical, microbial, and physical. Due to the high excretion of antibiotics into sewer systems, assessing their removal within wastewater treatment systems is vital to prevent their dissemination into natural water bodies. Sabri *et al., (*2020b) demonstrated that the treatment technology in question plays a key role in removing ARGs and antibiotics using detection methods such as quantitative PCR and liquid chromatography-mass spectrophotometry (LC-MS). The mechanisms involving their removal relate to intrinsic and extrinsic factors related to the antibiotics themselves (chemical structure) and their sorption to solids and filter materials involved in the treatment process. Including additional treatment steps is important in effectively removing antibiotics and ARGs from wastewater. Their study revealed that cost-effective aerobic granular sludge treatment can remove antibiotics from wastewater effectively compared to conventional activated sludge and filtering (Sabri *et al*., 2020b).

The activated sludge environment harbors a wide variety of microorganisms and microbial processes. In a study by Dai *et al.,* (2022), the activated sludge process can reduce the abundance of ARG in both chromosomal DNA and mobile genetic elements (MGEs) such as plasmids. The percentage abundance of ARG's associated with MGEs versus non-MGE associated ARGs increases in the sludge. Removal of key parameters such as suspended solids is once again highlighted as an important target for ARG removal due to the protection they offer during the treatment process and in natural environments (Sabri *et al*., 2020a, Sabri *et al*., 2020b).

2.1.4 Conventional and non-conventional treatment of antibiotics and ARGs in the rural context

Various challenges in South Africa led to the failure of wastewater treatment facilities such as sewage loss before treatment, vandalism theft, training constraints, construction and quality standards not being met, lack of commitment and participation by municipalities and funding constraints (DWS, 2023). It is thus crucial that alternatives be considered by policymakers and investigated by scientists of various disciplines in the implementation of new equipment and planning on simple, cost-effective, and low maintenance wastewater treatment systems. In rural areas within Vhembe, the close dependence of humans and animals on the environment exposes them to pathogens from sources such as sewage and greywater (Traore *et al.,* 2016; Potgieter *et al.,* 2020). Thus, cost-effective, sustainable, and value-producing treatment alternatives may offer a more holistic solution. Different methods exist for the conventional and unconventional treatment of wastewater. Conventional methods are typically mechanized to a greater degree, requiring more electricity, starting resources and maintenance. Non-conventional methods rely on more passive and natural processes to achieve treatment, often on sites known as decentralized wastewater treatment systems (DEWATS) (Fahad *et al.,* 2015). Constructed wetland (CW) technology is a non-conventional wastewater treatment involving mimicry of the wetland ecosystem to purify water. It shows good potential due to its advantages in cost, ease of operation, and high effluent quality. Although CWs can effectively remove bacteria and antibiotics from different wastewater, a major challenge CWs face is the lack of standardization in design. Optimization of system design may help mitigate other challenges, such as clogging and large area requirements. CWs also effectively lower important parameters contributing to ARG survival and transport, such as TDS with high values (98%), when combined with other treatment methods, such as oxidation ponds, for applicability in rural settings (Wang and Chen, 2022; Moreira and Dias, 2020).

2.2 Antibiotic resistance in bacteria

2.2.1 Antibiotic resistance mechanisms

Chemically, antibiotics can be divided into 11 classes: aminoglycosides, β-lactams, macrolides, oxazolidinones, glycopeptides polymyxins, tetracyclines, quinolones (fluoroquinolones), streptogramins, sulfonamides and others (a group of compounds including thiamphenicol, chloramphenicol, trimethoprim, lincomycin, and clindamycin) (Hutchings *et al,* 2019). Antibiotic resistance naturally occurs as microorganisms compete for resources, leading to "self-resistance" against antimicrobial compounds, some of which are transferrable. Peterson and Kaur (2018) categorized antibiotic resistance mechanisms into six main types based on their mechanisms of action (Table 2.2). These mechanisms can be intrinsic or acquired through MGEs, such as plasmids, enabling global dissemination.

Table 2.2 Mechanisms of antibiotic resistance (Adapted from Peterson and Kaur, 2018)

Mechanism	Description	Gene examples
Antibiotic modification	Addition of functional groups by various transferases (acetyl groups by AAC, phosphates by APH and adenyl groups by ANT) to alter antibiotics.	BlmB and TlmB,
Antibiotic degradation	Degradation of antibiotics by various enzymes by hydrolysis (Example B-lactamases)	*blaOXA*
Antibiotic efflux pumps	Physical "pumping" of antibiotics out of the cell using either ATP-driven efflux pumps or proton gradients	SMR (*S. aureus*) and EmeR (*E. coli*), *drab, OtrC, OtrB*
Target modification	The Modification of the target of a specific antibiotic, such as methylation of 16S or 23S ribosomal RNA (rRNA)	*vanH, van A, vanX* clusters (Vancomycin resistance)
Antibiotic sequestration	Proteins are synthesized by bacteria that can bind to antibiotics and allow for their removal	*TlmA, BlmA, and ZbmA*
Target bypass	Generation of "dummy targets" for antibiotics to bind to instead of the normal target (for example, addition of B-subunit in DNA gyrase for novobiocin to bind	*DrrC, OtrA*

* Key: AAC= N-acetyl transferases; APH= Phosphotransferases; ANT= O-adenyltransferases

2.2.2 Transfer mechanisms of antibiotic resistance genes within the wastewater environment

It is crucial to know the transmission of ARGs and their movement within humans, animals and the environment. ARGs are naturally acquired by mutations, horizontal gene transfer (HGT) (transduction via phage and conjugation) and vertical gene transfer (binary fission) (Wang and Chen, 2022). The survival of genetic vectors such as plasmids and phages have been highlighted in wastewater and the agricultural environment using advanced approaches such as metagenomics (Dai *et al*., 2022; Zhang *et al.,* 2023). Over the years, more data has shown some chromosomal genes are ARGs and may be mobilized by MGEs, facilitating their spread. ARGs found on either plasmids or chromosomes have been observed using metagenomic approaches in wastewater and activated sludge, suggesting that although significant reduction of ARGs within some wastewater facilities occurs, they may be mobilized via suspended solids (Dai *et al*., 2022). Bacteriophages have been shown by Zhang and colleagues (2023) to contain many ARGs within river water polluted by various fecal sources like improperly treated wastewater, possibly containing antibiotics or ARGs.

2.2.3 Antibiotic resistance within the Vhembe District

Antibiotic resistance genes, like the pathogens they associate with, use the intricate relationship between humans, the environment, and animals to be transferred from host to host. Both pathogen and ARG transfer through the environment (water and soil), and contaminated food pose, a high economic and health burden (Velazquez-Meza *et al*., 2022). In rural areas like Vhembe, close contact with livestock, crops, and wastewater-influenced rivers makes studying antibiotic resistance crucial due to the presence of various enteric pathogens in natural water sources, which could be transmitted via fecal-oral route (Gumbo *et al.,* 2010; Traore *et al*., 2016; Potgieter *et al*., 2020). Considering this interface, the One Health approach has been proposed as a holistic solution globally. It can be applied in rural settings to identify transmission between animals, humans, and the environment (Purohit *et al.,* 2017; Gholizadeh *et al.,* 2023). Reports of antibiotic resistance in Vhembe are limited and widespread over time (Samie *et al*., 2007; Obi *et al*., 2007; Tanih *et al*., 2015; Rakhalaru *et al*., 2023). HIV prevalence and dependence on polluted water sources for various activities may exacerbate the transmission and impact of both ARG and their bacterial host in Vhembe (Samie *et al*., 2007; Gumbo *et al.,* 2010; Traore *et al.*, 2016). In a review by Abia and colleagues (2023), studies on antibiotic resistance in South Africa are growing, however, there are still gaps in South Africa concerning the ARG dissemination in wastewater across the entire country, more so in rural areas.

2.2.4 Combating antibiotic resistance within rural communities: A wastewater perspective

The growing global concern for antibiotic resistance is greatly realized in rural settings where people, animals and the natural environment rely more directly on one another. This is due to factors such as inadequate wastewater treatment infrastructure, lack of local knowledge of pathogen and ARGs transmission and overuse/misuse of antibiotics in humans and animals (Abia *et al.,* 2023). Sewage surveillance may be necessary as a preliminary step to combating ARGs by essentially posing these questions: What is circulating in the community (influent WW), and what is being introduced to the environment beyond the treatment plant (effluent WW)? Several surveillances have been done in South Africa in relation to ARGs and bacteria using genotypic (NGS, PCR), phenotypic (biochemical testing), and culturing methods. Some include antibiotic resistance to various antimicrobials in *Vibrio* species (Okoh and Igbinosa, 2010), *E. coli* (Odadjare *et al*., 2012; Pillay and Olaniran, 2016), and *Enterococcus* in areas with varying levels of urbanization (rural, peri-urban and urban) (Hamiwe *et al*., 2019).

Makuwa and colleagues (2023) revealed considerable bacterial diversity within effluents and influents in municipal wastewater using sequencing approaches. Species varied from pathogenic ones causing diseases such as gastroenteritis, legionellosis, tuberculosis and *Staphylococcus*/*Streptococcus* infections; and those capable of nutrient removal, such as phosphate removal (*A. calcoaceticus, A. hydrophila P. fluorescens, S. aureus*), sulfate removal (*Desulfovibrio* species) and denitrification (*R. capsulatus*,). Using molecular methods such as NGS poses challenges in rural settings, such as cost and training requirements. However, it is becoming evident in Africa that AR studies deploying molecular methods capable of detecting multiple targets are being conducted in rural settings (Abia *et al*., 2023)

Within a rural setting, ARB is closely linked with one of the largest consumers of antibiotics: animals (and thus animal WW), posing high risks for ARG transmission. Antibiotic resistance has been investigated in wastewater from animals using One Health, revealing a wide array of gram-negative bacteria. Amongst them are pathogens such as *E. coli* (multiple pathotypes) and non-*E. coli* coliforms (Purohit *et al*., 2017; Akpan *et al*., 2020). Decentralized WW treatment systems, such as constructed wetlands, show promise in these settings regarding cost and pollutant removal efficiency, given that the clogging challenges are mitigated (Moreira and Dias, 2020).

A review by Lee and colleagues (2013) outlined several factors important to combating AMR:

a) antibiotic prescription regulation and revision of antibiotic treatment guidelines,

b) antimicrobial stewardship programs.

c) education of clinicians continually concerning antibiotic resistance, and virulence and passing of knowledge of ARGs to the public,

d) hygiene and disinfection with special attention to nosocomial infections, handwashing, surface disinfection and wearing of protective equipment in the reduction of pathogenic bacteria such as *C. difficile,* and *Acinetobacter species*,

e) veterinary medicine, whereby the use of closely related antibiotics in humans and animals may confer resistance to human pathogens and the transfer of genes via HGT play a role in ARG transfer. This may be mitigated by improving disease prevention via hygiene and vaccine and using alternative treatments such as phage, bacteriocins and probiotics,

f) Novel antibiotic discovery.

The latter point is an increasingly important driving force. Novel antibiotic discovery is decreasing with an increase in antimicrobial resistance (AR). Additionally, new compounds (oxazolidinones, cyclic lipopeptides; pseudomonic acids and pleuromutilins) are less broadly reactive. One major challenge with novel antibiotic discovery, however, is cost, which may require new business models and political action, such as sharing research costs with large pharmaceutical companies and tax breaks to increase discovery (Lee et al., 2013).

Because, in rural settings humans, the environment and animals often live in close tandem, some challenges may be more achievable by targeting local policymakers, schools and healthcare workers to further their knowledge on AR and subsequently educate the local community through collaboration with local institutions and business.

2.3 Methods for detecting and characterizing microbes and ARGs in wastewater

2.3.1 Detection methods for bacteria and ARGs in wastewater and sewage impacted surface water.

Several methods exist for detecting antibiotic resistance genes in humans and the environment. Molecular techniques like PCR are the gold standard for ARG detection. PCR involves the cloning of target genes using oligonucleotide primers through three main stages of varying temperature: denaturation of double-stranded DNA, annealing and extension. It can, for example, detect resistance genes in wastewater associated with various resistances against different antibiotics (Ziembińska-Buczyńska *et al.,* 2015).

Another approach is metagenomic sequencing, which was first mentioned in 1998, a high throughput approach which can analyze multiple genomes without the need for culturing (Handelsman *et al*., 1998). It employs various bioinformatics pipelines to characterize data, such as assigning taxa to sequences and procuring gene subsets, MGEs and bacteria. Metagenomics is a powerful tool for not only detecting ARGs and microorganisms but also for determining the selection of certain genes during wastewater treatment (Bengtsson-Palme *et al.,* 2016).

Traditional culturing methods for determining AR involve techniques such as the Kirby-disk-diffusion method and broth dilution. They both offer the advantage over molecular techniques in that they offer determination of phenotypic resistance, however, genes can be activated or deactivated and essentially lying dormant until needed. This leads to a discrepancy between genotypic resistance and phenotypic resistance whereby resistance genes may be detected but not expressed, as seen in the study by Van den Honert and colleagues (2021) on samples from wild and domestic animals. Phenotypic resistance, however, may not be because of one gene but many working in tandem, as suggested in a study by Govender and colleagues (2021) on *Aeromonas* and *Pseudomonas* species in wastewater and river water. Additional disadvantages of culturing methods are their high time consumption and labour demand.

2.3.2 Determination of antibiotic resistance and ARGs in wastewater in South Africa

Identifying which ARGs are prevalent in the environment and wastewater is vital for predicting and addressing AMR across communities. A recent review by Abia and colleagues revealed the typical methods being used to determine AR as molecular (PCR, whole genome sequencing and shotgun metagenomics), biochemical (VITEK 2) and culturing techniques (Kirby-Bauer disk diffusion). They highlighted the factors that influence the choice of method, including the cost, labour intensity and contamination risk. Furthermore, the effectiveness of studies is impacted by factors such as sampling infrequencies, equipment cost and research funding. In this light, automated systems like VITEK 2 and shotgun sequencing could allow communities to adequately generate resistome data on a wide variety of bacteria and their associated elements. Potential data in settings such as rural, urban, peri-urban and the wilderness within the Vhembe region may be attractive for future investigations into resistomes within the environment, animals, and humans.

2.4 SUMMARY OF THE LITERATURE

Wastewater treatment plants allow for the dissemination of antibiotics and ARGs into the environment. Antibiotic resistance is acquired by introducing low quantities of antibiotic-derived metabolites to bacteria within the environment. ARGs have different survival rates depending on the gene and exhibit seasonality, surviving long distances after effluents and into natural water bodies such as rivers. ARGs are persistent, and their removal is dependent on different factors, both intrinsic and extrinsic, to the treatment plant, such as the secondary or tertiary treatment technology used and the presence of extrinsic influences such as the ARG in question and the removal efficacy of various nutrients and suspended solids. Activated sludge process and modified trickling filters are the most effective secondary treatment. Various studies show that the addition of a tertiary treatment aids in removing ARGs and ARBs.

Antibiotic resistance is obtained through the development of different strategies by the bacteria to prevent antibiotics from affecting them. These mechanisms include efflux pumps, modification and degradation of target antibiotics, modification of antibiotic targets and generation of “dummy targets”. Antibiotic resistance genes can be transferred in various ways: transduction via lysogenic phage uptake of MGEs or mobilization of chromosomal ARGs via plasmids. In rural areas, humans and animals use water polluted by various human activities and domestic sewage and are exposed to various pathogens, including bacteria. Reports of AMR in Vhembe are scarce. They are predominant in clinical studies, highlighting the need to examine ARGs due to high levels of water pollution and the prevalence of HIV in the region.

The gold standard for studying ARGs in the environment is PCR, which has successfully detected ARGs in different aquatic environments across South Africa. These methods are cost-effective but have a limited scope of ARGs they can detect at a time. Metagenomic sequencing techniques have demonstrated the detection of a wide spectrum of ARGs and their various carriers, such as plasmids and phages. These tools would be useful in rural settings to determine the presence of a large variety of bacteria and ARGs.

Chapter 3: Methodology

3.1 Study site:

This study was conducted in the Vhembe region (Figure 3.1), Limpopo Province, in the Northern South Africa. The district covers an area of 25,597 km$^{2.}$ The Vhembe District has 16 WWTPs. A total of ten sites were selected for this study.

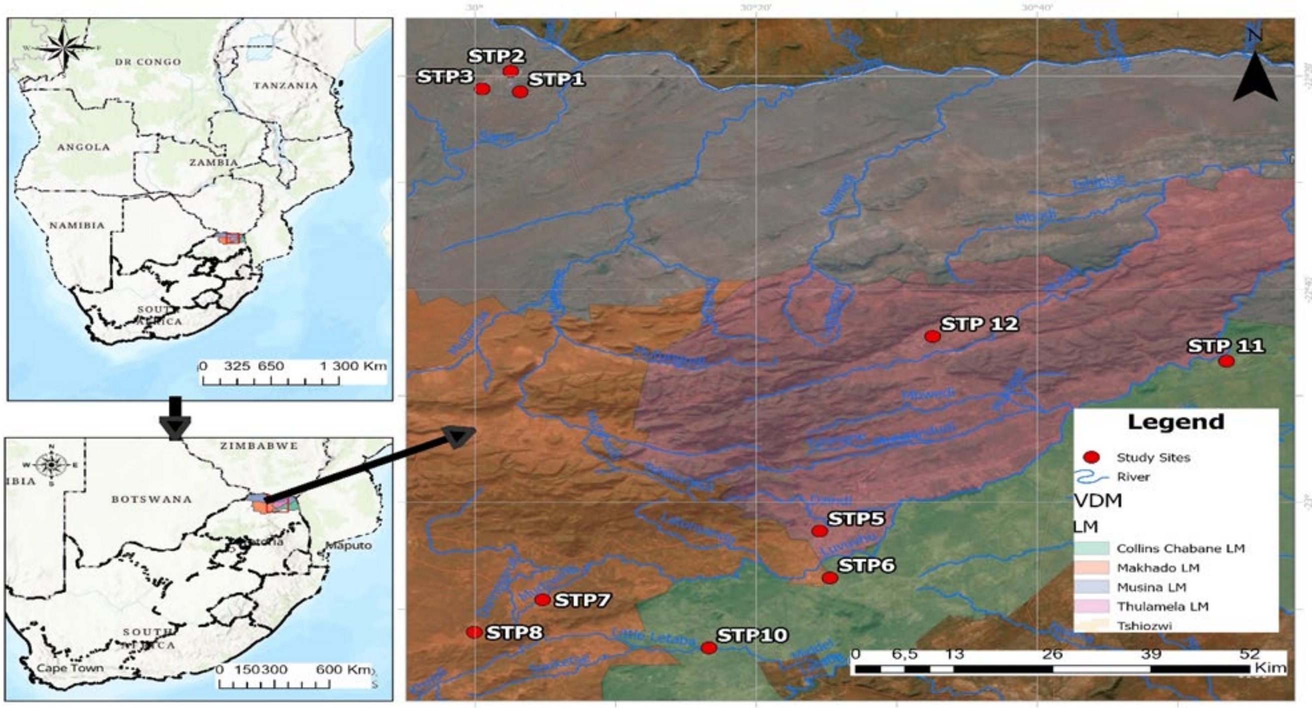


Figure 3.1: Geographical map of the Vhembe region, Limpopo, South Africa, with pins on the WWTPs selected.

3.2 Ethical clearance

Ethical clearance to conduct this study was obtained from the University of Venda Ethics Committee, and permission to collect samples was approved by the wastewater treatment plants' management.

3.3 Sample collection.

A total of 32 duplicate sample were collected in October 2023 from all nine WWTPs from the influents (n = 18) and final effluents (n = 14). Each sample was collected in 250 mL sterile collection bottles and transported on ice to the University of Venda Water and Health research laboratory for processing. Structured observations were performed using a short observation sheet/questionnaire (Appendix A) to gather information on some activities in and around the plant.

3.4 Sample analysis.

3.4.1 Membrane filtration and DNA extraction

Membrane filtration was performed according to Ekwanzala and colleagues (2020) with slight modification. Briefly, 100 mL of each sample was passed through sterile cotton gauze and then Wattman1 filter paper to remove large debris and eukaryotic cells. The samples were subsequently filtered through a sterile 0.22 µm membrane filter (Millipore, Merck, South Africa). The 0.22 µm membranes were snipped up using flame-sterilised scissors and directly used for DNA extraction using the ZymoBiomics DNA miniprep kit (ZymoBIOMICS™, Zymo Research, California, USA) according to the manufacturer’s instructions. The DNA was quantified using the NanoDrop™ 2000 spectrophotometer (Thermo Scientific, South Africa). The extracted DNA was shipped on Ice to the National Centre for Communicable Diseases for Metagenomic sequencing. The Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA) was used for library preparation. Illumina NextSeq Machine (Illumina, San Diego, CA, USA) was used to conduct NGS shotgun metagenomic sequencing.

3.4.2 Bioinformatic analysis

Raw sequencing files were uploaded and analysed using the Chan Zuckerberg ID (CZ ID) portal (https://czid.org) open bioinformatics pipeline, under default settings. The pipeline performs data quality control, adapter trimming and host DNA subtraction. Alignments using Bowtie 2, which matched raw fastq files reads to the national nucleotide collection of the National Center for Biotechnology Information (NCBI) (BLASTN and BLAST) for species identification. ARG analysis was further performed using the CZID Resistance Gene Identifier tool, which compared quality-controlled reads and assembled contigs against the Comprehensive

Antibiotic Resistance Database (CARD). Flower plots and network analysis were done online using Evenn (Chen et al., 2021). Heatmap generation and principal component analysis (PCA) were done using ClustVis (Metsalu and Vilo 2015).

3.5 Summary of the Methods

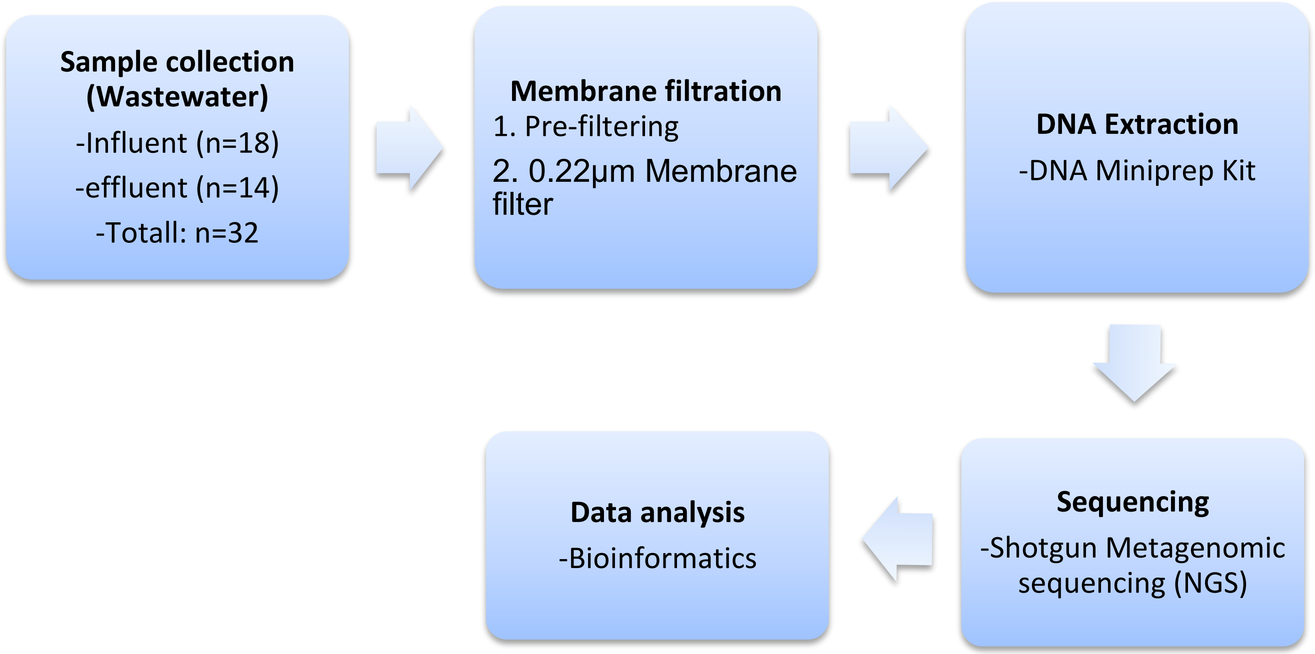


Figure 3.2: Flow chart of methodology

3.6 Optimization of the methods

To optimize the isolation and extraction efficiency. A duplicate sample of raw sewage (n=2) was collected from a random manhole from the University of Venda, Thohoyandou. Samples were filtered according to the methods mentioned above in section 3.4.1. Table 3.1 illustrates the results of the DNA analysis from the 2 raw sewage samples obtained.

Table 3.1: DNA quality and quantity obtained from the duplicate sample.

Sample ID	Quantity (ng/µL)	A260/280	A260/230
S1	55.6	1.96	1.06
S2	64.4	1.90	1.29

Chapter 4: Results and discussion

4.2 Results

4.2.1 Site observations

Some observed WWTPs across Vhembe are either vandalized or in disrepair (Fig 4.1, 4.2, 4.3). Even some sites which are regularly operated such as those in Musina are challenged with vandalism by elephants as reported by some workers at the sites. Some sites such as the latter are located within arid areas and may be attractive to various animals in the area due to the constant supply of water. A notable lack of maintenance or the influence of large animals such as cows and elephants, which frequent some sites in search of water and grazing, allows the plants to be frequented by humans and smaller animals such as sheep or antelope.

Figure 4.1: Images of a vandalized wastewater treatment pond facility with damaged buildings (top left), one dry oxidation pond (top right), a functioning pre-treatment (bottom left) and a sparsely filled oxidation pond (bottom right)

Figure 4.2: WWTP showing oxidation ponds with various activities of animals such as cows, crocodiles (top right), and birds (top left, top right bottom right), and an offsite commercial meat chicken farm across the road (middle left) and the final oxidation pond containing an algal bloom (middle right). Human activities such as fishing (bottom left)

Figure 4.3: Abandoned WWTP showing wetland growth in oxidation ponds (Top left, lop right) Dried inlet and alternative inlet (Middle left and middle right) and the visible difference in turbidity from influent to effluent.

4.2.2 Activities around the treatment plant

All plants showed some degree of animal activity (Table 4.1). The most prominent feature around the sampled WWTPs was the diversity of animals that frequented the sites during sample collection. Animals were observed at all sites, ranging from domestic livestock such as cows, pigs, and sheep to wild animals such as crocodiles, elephants and various birds such as the sacred ibis, Egyptian geese, grey heron, egrets and guinea fowl. Some WWTPs which the animals visit receive wastewater from multiple settlements and some from hospitals too (Table 4.1). Effluents were also indicated to enter various rivers that permeate across the region. Some sites (STP 5 and STP 8) had no effluent points and others had effluents which flowed to outside the parameters. These effluents are still exposed to the community and are likely utilized for their nutrients in some farming activities. Cows and sheep which local community members keep were observed on-site to drink from the oxidation ponds and final effluents in plants spread across the region (Fig 4.2). Pigs and warthogs were reported to frequent the sites, and STP 11 showed evidence of porcine activities within the plant itself. Animals also influenced the integrity of the plants themselves; elephants were reported by site staff to frequent the sites in the Musina area, damaging fences (STP 1, STP 2, STP 3). Other sites such as STP 5 and STP6, also had damaged fences, cattle being the largest animal frequenting the site, which may have caused the damage. The most common secondary treatment used across Vhembe was oxidation pond (Table 4.2). Safety gear was worn at all WWTPs, and all but one (STP12) reported eating their food on site. STP 12 also had an ill worker on site, reporting flu symptoms. Four of the plants discharged their effluents into rivers across the region (Table 4.1). The predominant treatment technology was oxidation ponds, with tertiary treatments being chlorination in some plants (Table 4.2).

Table 4.1 Animal presence in and around the WWTPs based on data collected from filled forms.

	Animals encountered			No. of Hospitals/clinics supplying WW	No of settlements supplying WW	Receiving river	Setting		
Sample ID	**Cows**	**Birds**	**Other***				**Rural**	**Urban**	**Peri-urban**
STP1	-	✓	✓	1	2	Sand river	-	✓	-
STP2	-	✓	✓	1	4	Unknown stream	-	✓	-
STP3	✓	-	-	3	2	Sand river	✓	-	-
STP5	✓	-	✓	-	1	Dzondo Stream	✓	-	-
STP6	✓	✓	✓	1	-	Luvhuvhu River	✓	-	-
STP7	-	✓	-	-	2	Mudzwiridi	-	✓	-
STP8	✓	✓	-	-	-	Muhuhudi River	✓	-	-
STP10	✓	-	-	-	-	Klein Letaba river	✓	-	-
STP11	✓	✓	-	-	1	Luvuvhu river	✓	-	-
STP12	-	✓	✓	1	1	lumvulini stream	✓	-	-

*Other animals may include Wild birds, elephants, pigs, goats, warthog, horses, springbok and/or monkeys

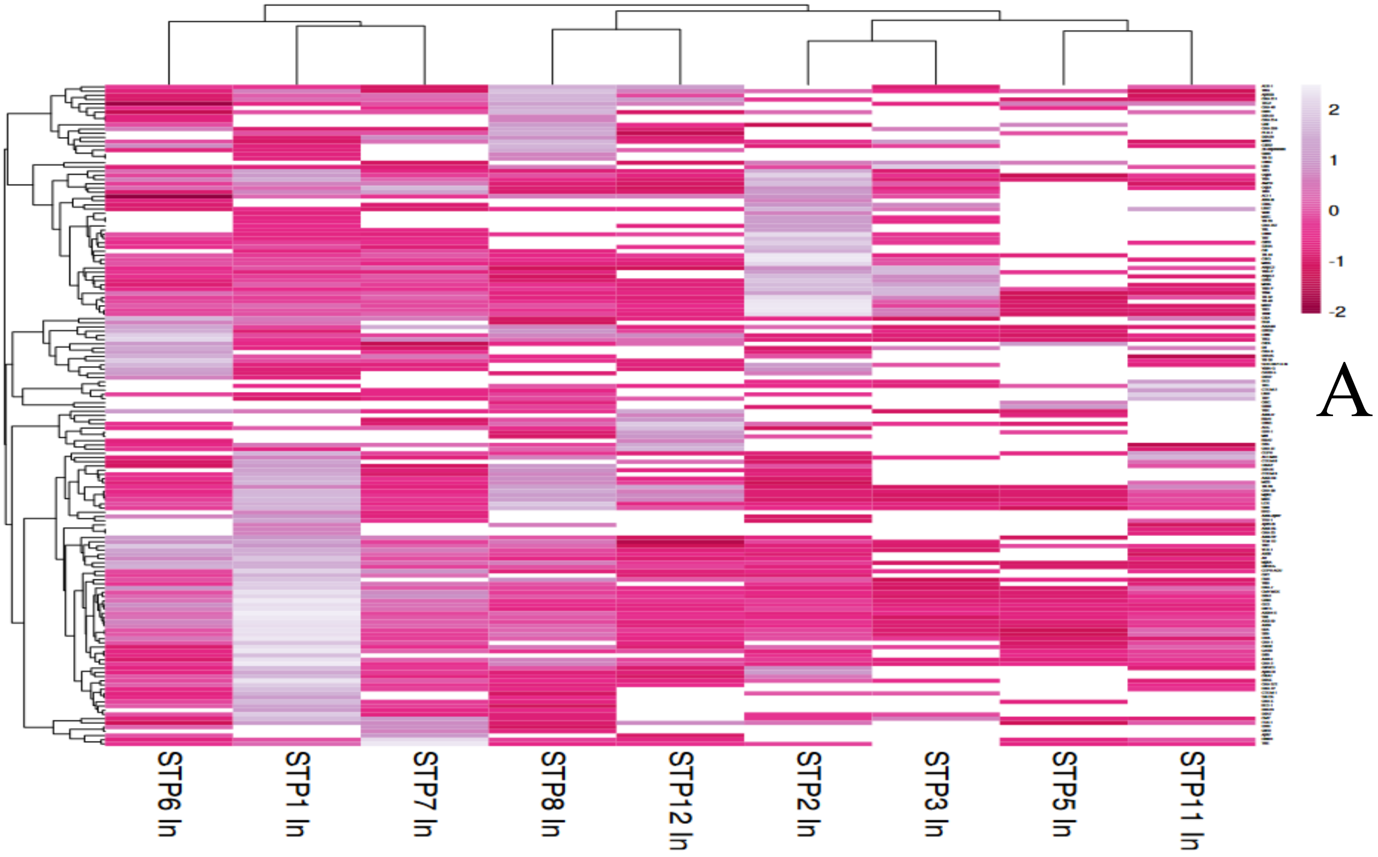
Table 4.2: Treatment techniques used by different sites.

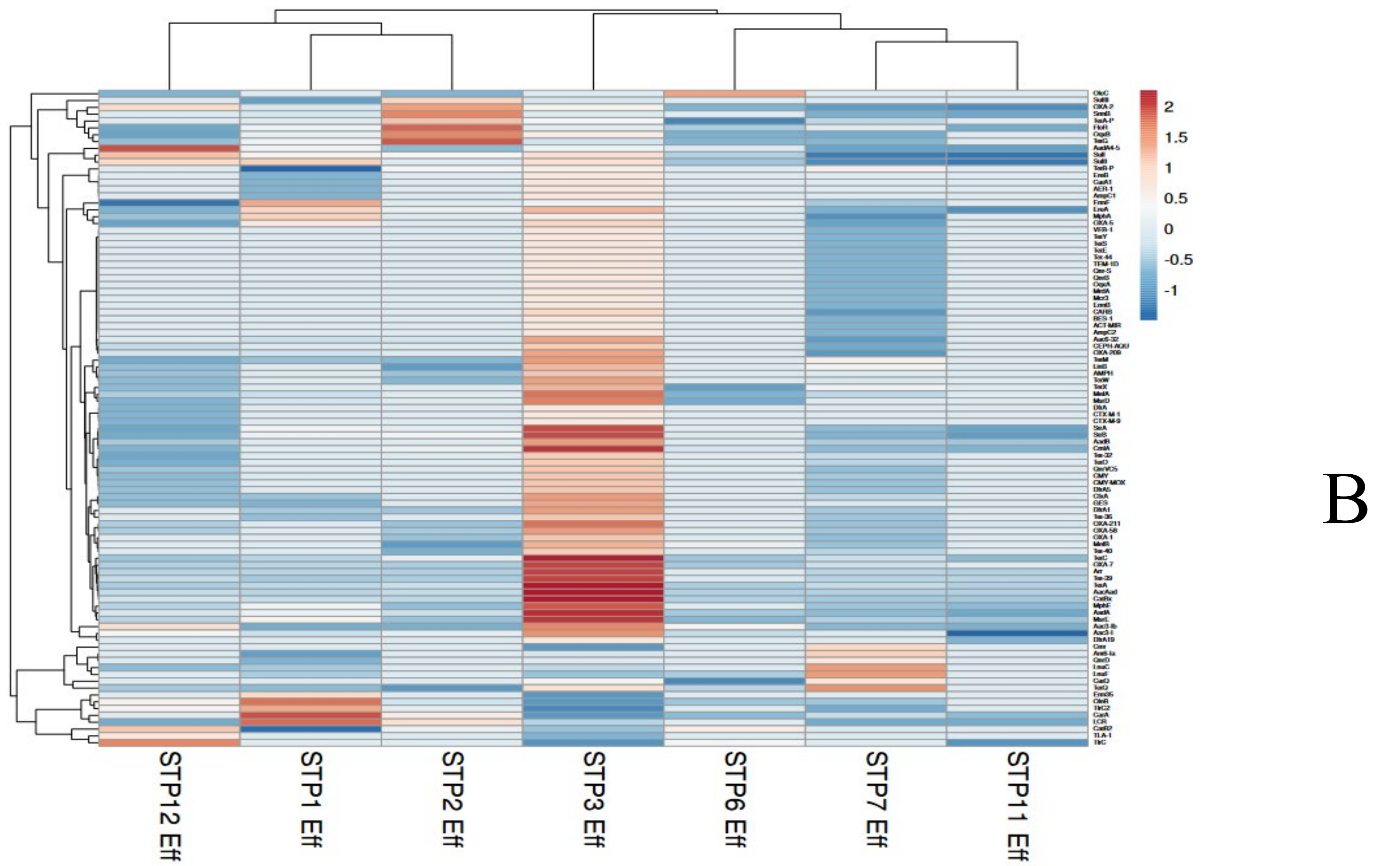
Sample ID	Primary treatment	Secondary treatment	Tertiary
STP1	Oxidation pond	Oxidation pond	-
STP2	Settling Tank	Oxidation pond	Chlorination
STP3	Settling Tank	Oxidation Pond	Chlorination
STP5	Oxidation pond	Oxidation pond	-
STP6	Oxidation ponds	Oxidation ponds	-
STP7	Clarifier	Clarifier	Chlorination
STP8	Oxidation pond	Oxidation pond	-
STP10	Oxidation pond	-	-
STP11	Oxidation pond (overgrown)	Oxidation pond	-
STP12	Oxidation pond	-	-

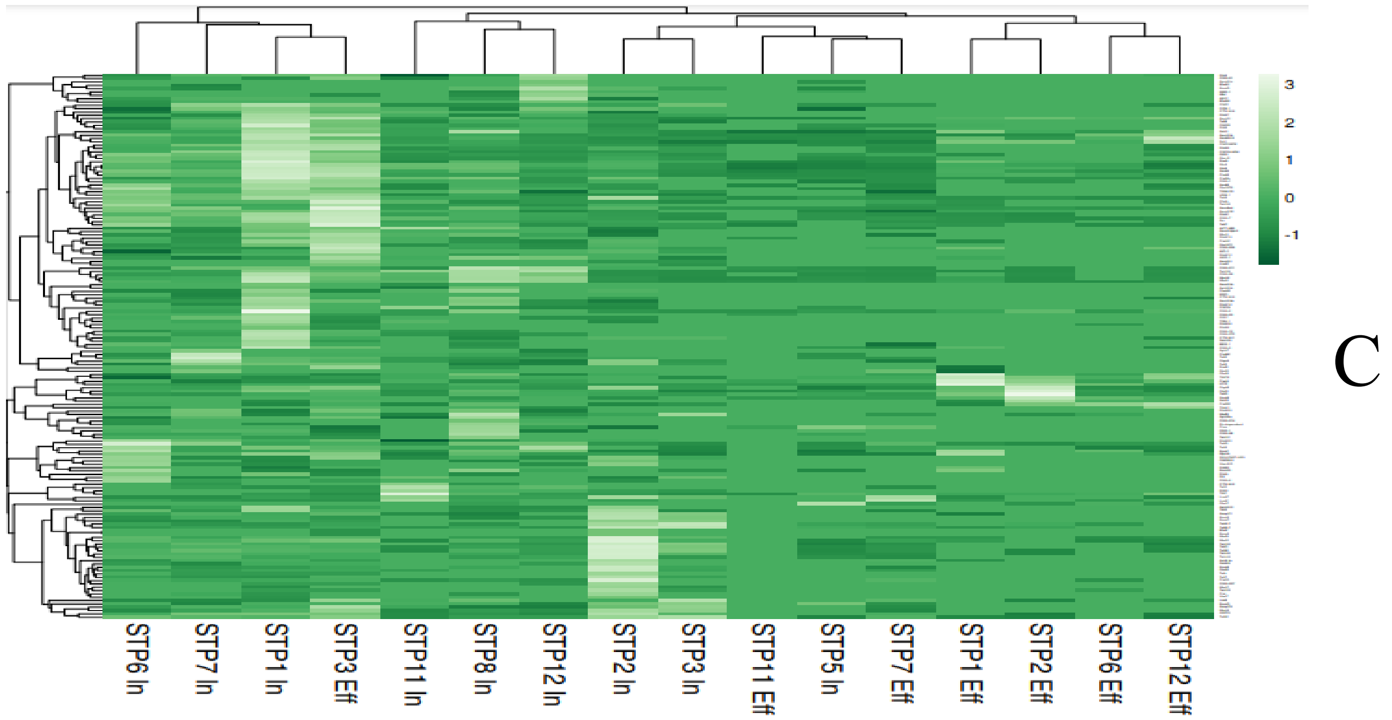
4.2.3 Antimicrobial Resistance Genes composition and diversity

Sixteen shotgun metagenomic pooled samples were sequenced at the NICD Sequencing Core, generating more than 20 Gb of unzipped data for processing. An average of 19252980 raw reads were obtained from the samples. Firstly, the ARG content of each matrix was explored, shotgun metagenomic sequences were queried against the CARD database using blastx to identify ARG sequences. This analysis revealed a wide range of ARGs in different assessed WWTPs, with totals of 68690 (influents) and 27787 (effluents) mapped reads and an average of 7632 (influents) and 3970 (effluents) mapped reads.

ARG diversity is shown by Fig. 4.4, 4.5, 4.6 and 4.7. Figure 4.4a denotes ARG composition and comparison. Relative abundances of ARGs profiles detected in assessed wastewater samples are represented in heatmaps of the influent (Fig 4.4A), effluent (Fig 4.4B) and across all WW samples (Fig 4.4C). Clustering of WWTPs across all samples (Fig 4.4C) shows clustering of influent versus effluent, except for STP3 effluent and STP5 Influent. Influent samples from STP5 and STP7 clustered showing they possessed a similar diversity. These plants are located approximately 50 km apart and service settlements under two different municipalities; however, STP5 was vandalized. The plants with the highest ARG diversity were STP1 Influent, STP3 effluent and STP8 influent. The most prevalent genes across all plants were *msrE, sulI, tetO* and *tet-39*. The most prevalent genes within the three plants were *msrE* (STP1 influent, STP8 influent), and *tetO* (STP2 influent). Other prevalent genes were *mphE*, *sulI/sulII*, *tetW*, *strB*, and *tetC*.




2
1.5
1
0.5
0
-0.5
-1
STP12 Eff
STP1 Eff
STP2 Eff
STP3 Eff
STP6 Eff
STP7 Eff
STP11 Eff
B


3
2
1
0
-1
STP6 In
STP7 In
STP1 In
STP3 Eff
STP11 In
STP8 In
STP12 In
STP2 In
STP3 In
STP11 Eff
STP5 In
STP7 Eff
STP1 Eff
STP2 Eff
STP6 Eff
STP12 Eff
C

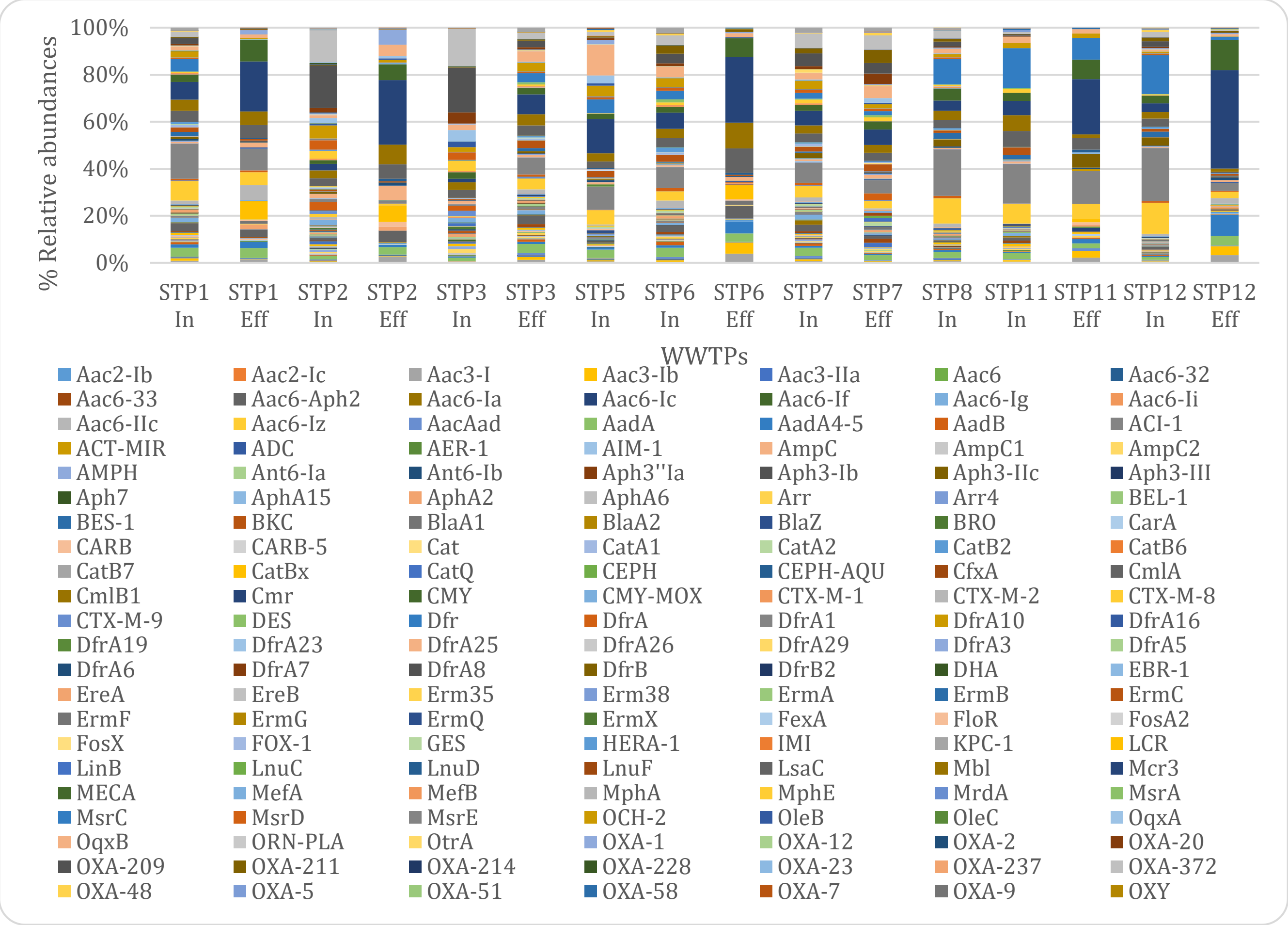


Figure 4.4 Heatmaps showing the relative abundance of ARGs in the influents (A), effluents (B) and all plants (C) of the selected WWTPs in the Vhembe District.

At the class level, based on functionality, the predominant type of ARGs across all samples were: antibiotic target protection (*msrE, tetO, tetW*), Antibiotic target replacement *(sulI, sulII*), antibiotic efflux (*tet-39, tetC*) and antibiotic inactivation (*mphE, strB*).. These genes encode for resistance against streptogramin (*msrE*), macrolides (*msrE, mphE*), tetracyclines (*tet-39, tetO, tetW, tetC*), sulfonamides *(sulI, sulII*) and aminoglycosides (*strB*). Sample dissimilarity in influents and effluents, respectively was analysed using PCoAs (Figs 4.5A and 4.5B). In the influent, STP1 and STP2 clustered separately from each other and the other plants. In the effluent, STP3 clustered on its own.

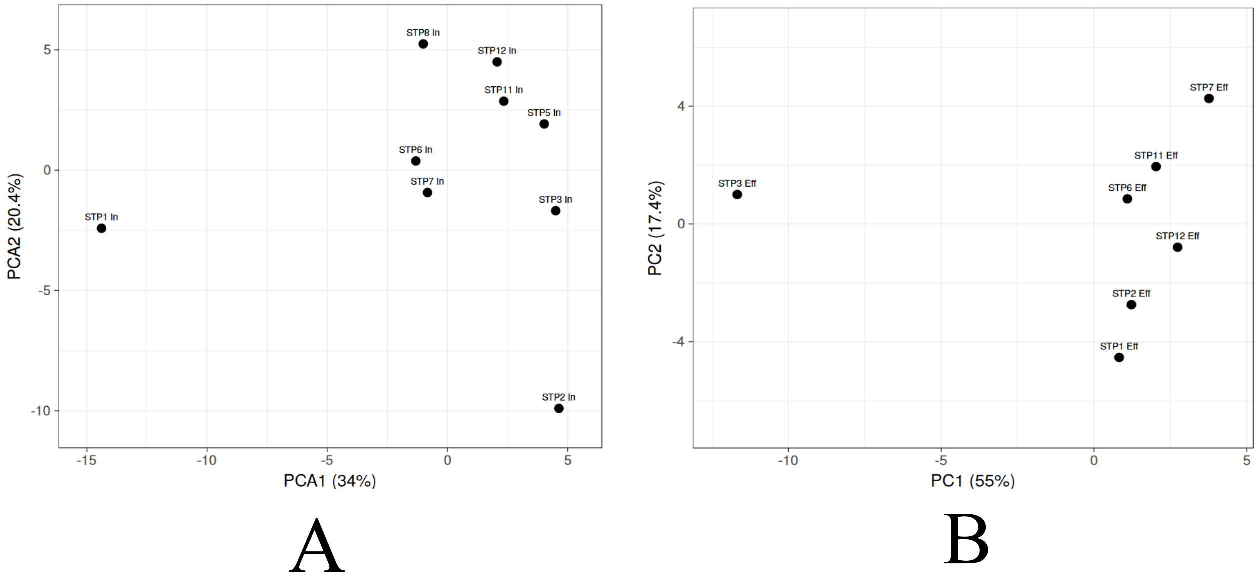


Figure 4.5: Principal component analysis (PCoA) of influents (A) and effluents (B) performed on the normalised resistome eigenvectors.

The number of unique genes that were detected per sample reveals that effluents of STP3 had the highest number overall and proportion when comparing to the other WWTPs (Fig 4.6A, Fig 4.6B). Most of those genes are encoded for mechanisms such as antibiotic inactivation, predominantly conferring resistances against aminoglycosides and β-Lactamases. Some genes conferred multidrug resistance such as *OXA-12, ACI-1, msrC and PER-1*. The second highest value is STP2 influents, with ARGs encoding antibiotic targ*et al*teration against glycopeptide ABs and multiple antibiotic resistance genes such as *ermA, vatD* and *HERA-1.*

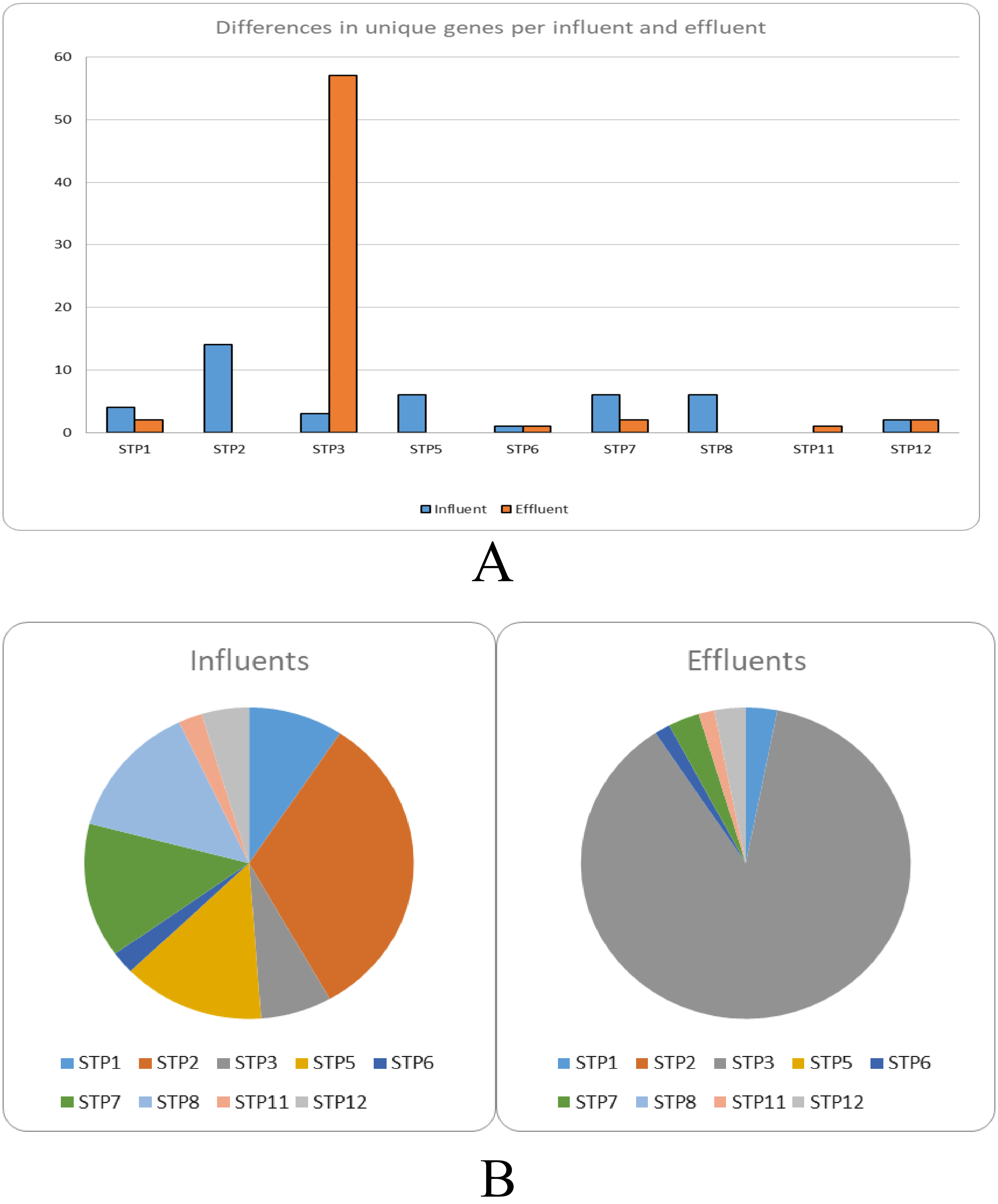


Figure 4.6: Proportion of unique genes across all samples (A), and in samples from the various sources B (i.e., influent and effluent). Example: *Aac6-Ig* and *OXA-228* were uniquely detected in the influent of STP12

In all assessed WWTPs, the core resistome was defined as shared ARGs found in the influents (Fig 4.7A) and effluents (Fig 4.7B). The influent core resistome was found to be 36 while the effluents had a core resistome of 16. The highest number of ARGs in influent was in STP 1, the lowest number was in STP 5. The highest number of ARGs in effluents is in STP 3 and the lowest number was in STP 11. The influent and effluent samples also shared common ARGs (Fig 4.8). Interestingly, one ARG, *carA*, was common amongst all effluent samples but not in the influent samples. Figure 4.9 shows the link between the various treatment plants regarding their ARG similarity in a network plot.

Table 4.3: Reduction of mapped reads in different treatment plants in Vhembe of most abundant ARGs

Gene name	STP1 In	STP 1 Eff	STP 2 In	STP 2 Eff	STP 3 In	STP 3 Eff	STP 5 In	STP 6 In	STP 6 Eff	STP 7 In	STP 7 Eff	STP 8 In	STP 11 In	STP 11 Eff	STP 12 In	STP 12 Eff
MsrE	2477	403	109	35	28	946	126	740	2	629	96	2037	901	46	1633	105
SulI	1260	928	225	889	50	1119	187	575	509	451	109	448	332	76	271	1280
TetO	431		1518		714	362	4	336		397	75	221	30		169	6
Tet-39	870	2	33	9	8	510	76	311		186	28	1122	920	30	1192	44

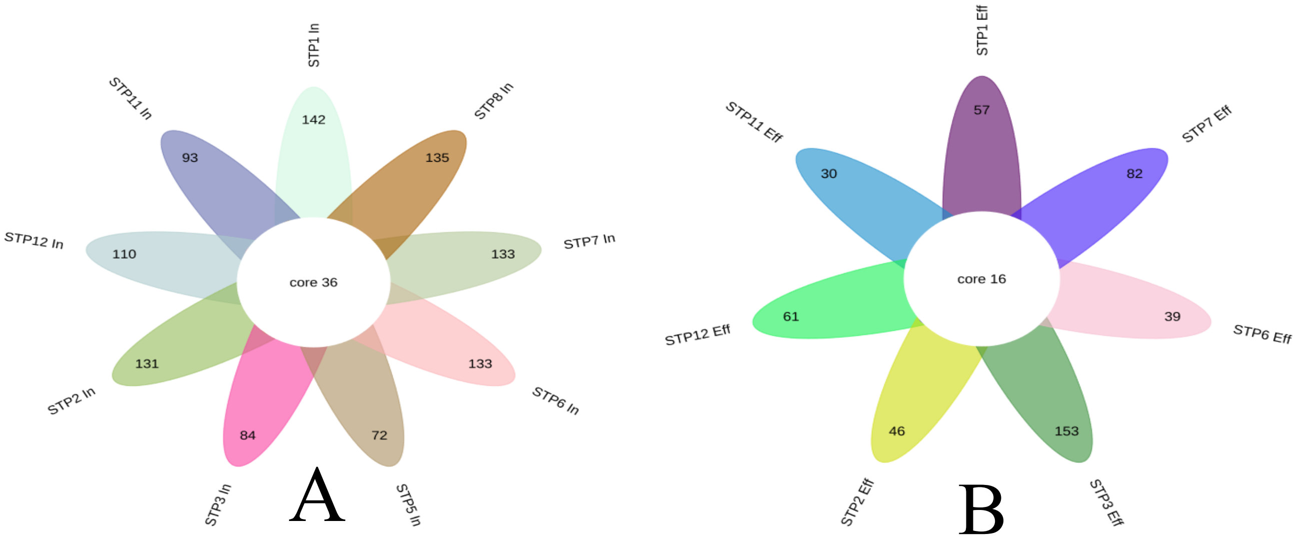


Figure 4.7: Flower Plot shared genes between the influent (A) and effluent (B). Each petal denotes the number of different ARGs detected in each plant and the core denotes the number of genes commonly found across all influents or effluents.

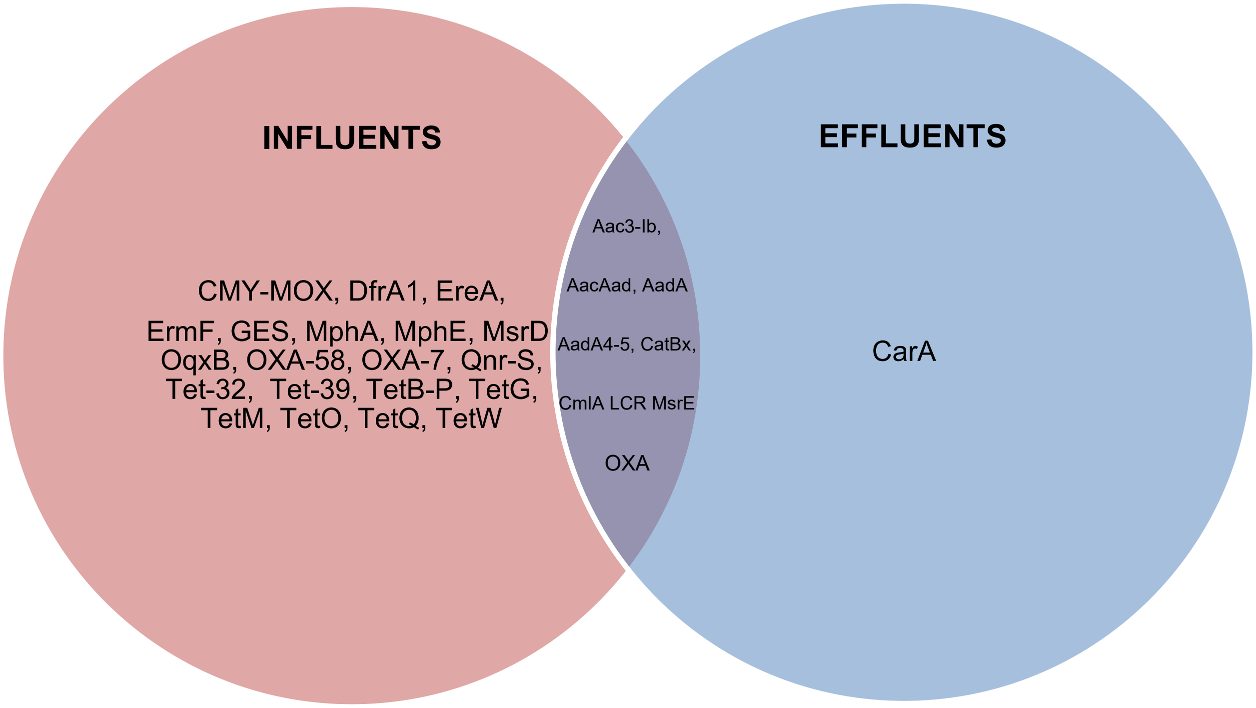


Figure 4.8: Venn diagram of the ARGs most common across all samples (i.e. Detected in all 16 samples) between the influents and effluents. *carA* (beta-lactamase resistance) was common across all effluents but not influent samples.

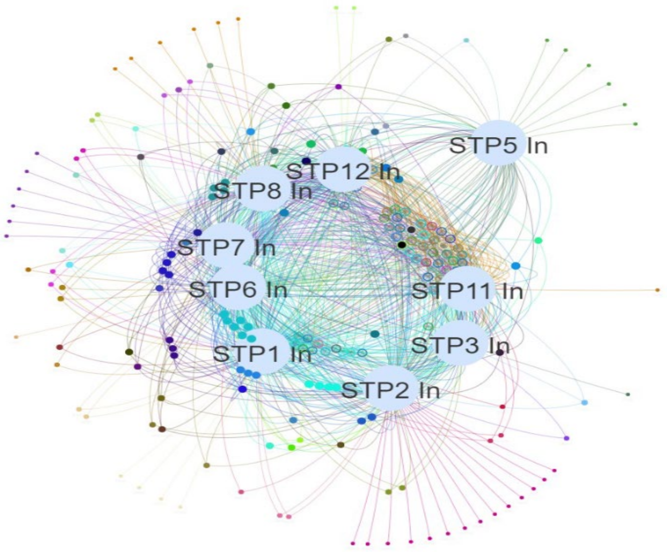
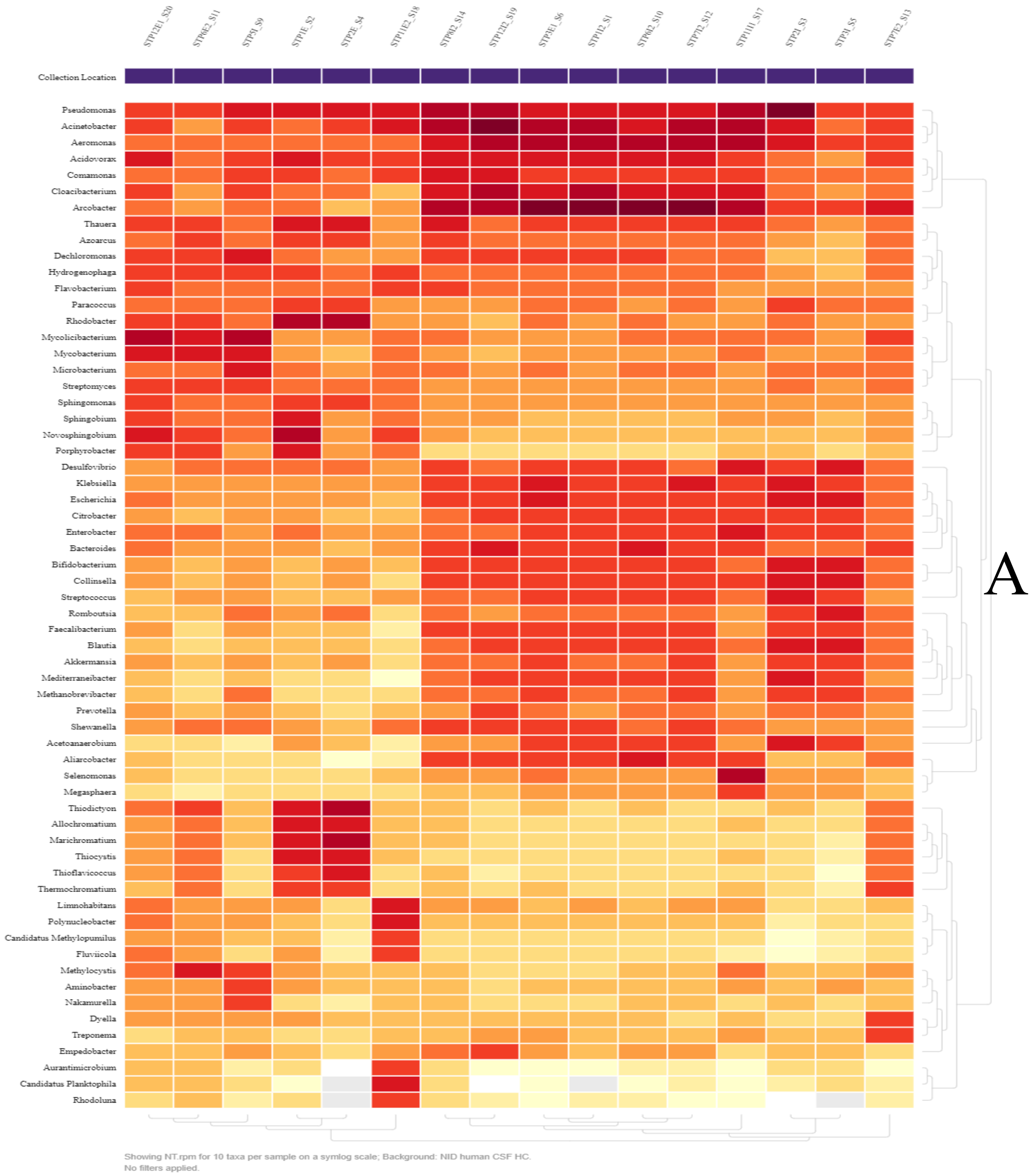
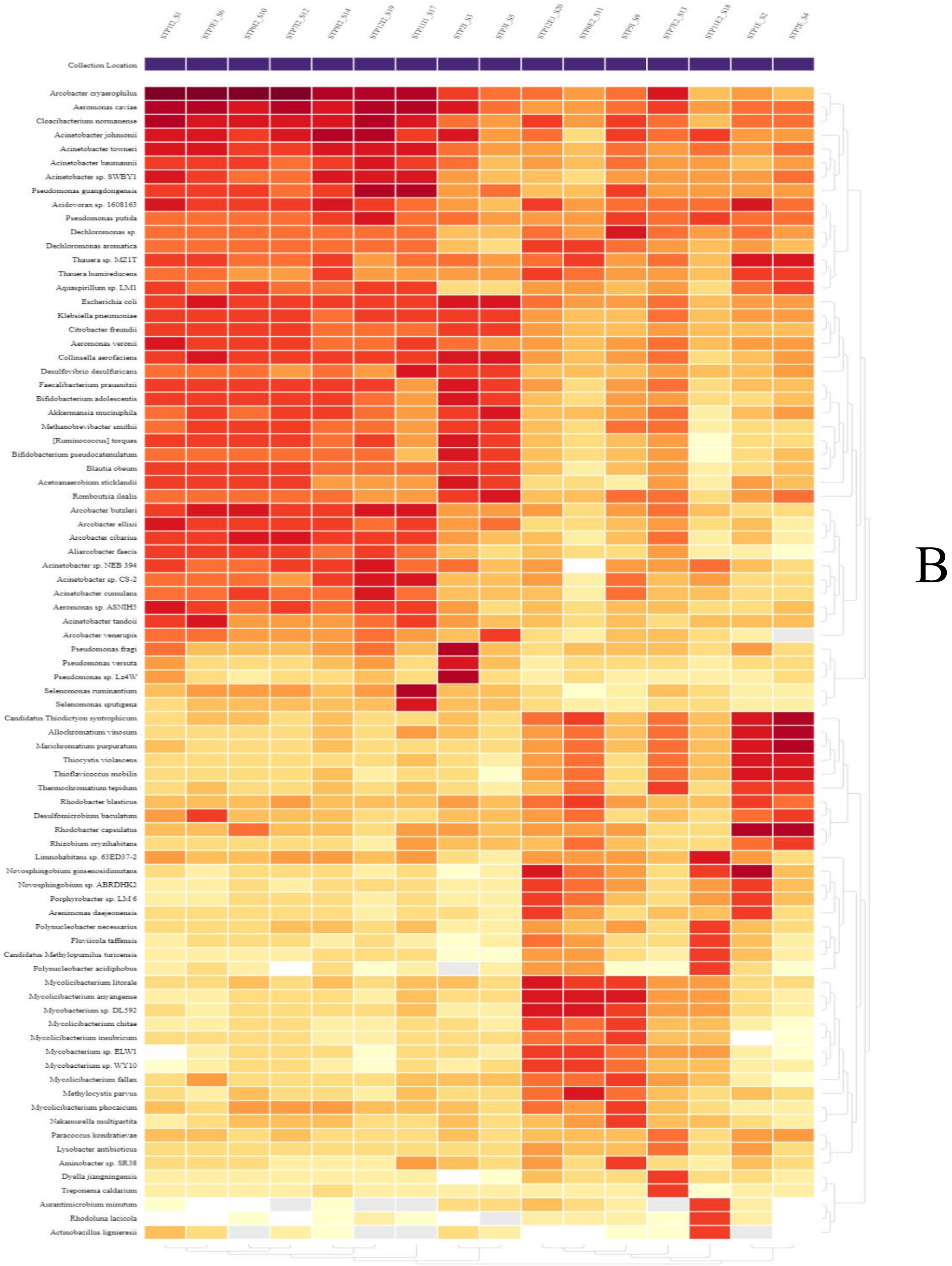


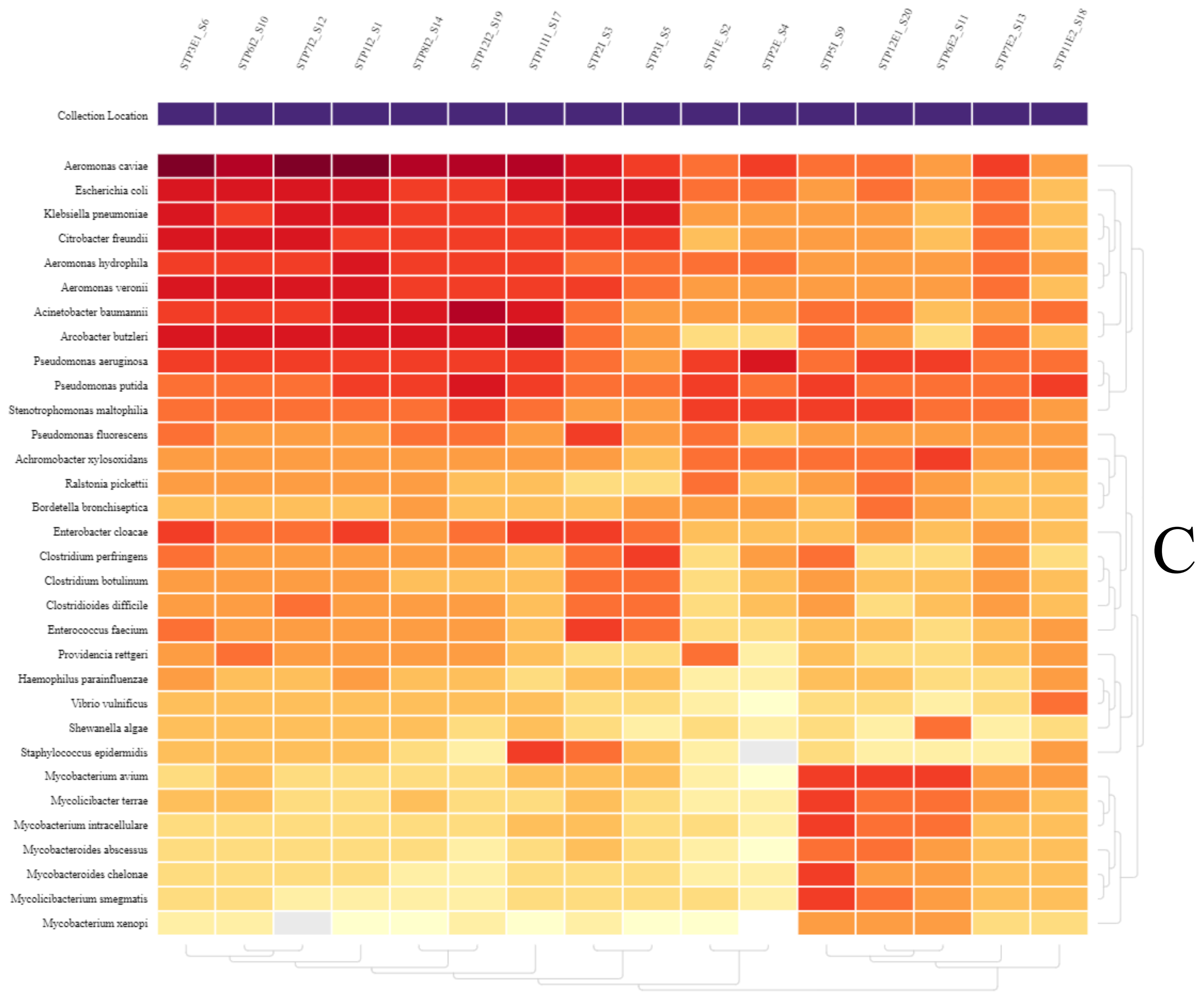
Figure 4.9: Network plot denoting the link between the various sites regarding similar genes circulating in the community.

Analysis of the bacterial composition and diversity revealed diverse genera (Fig 4.10A), and species (Fig 4.10B). Known bacterial pathogens were also identified (Fig 4.10C). On the genus level (Fig 4.10A), there is some level of hierarchical clustering between effluents and influents. (except STP5 influent, STP7 effluent and STP3 effluent). The plant with the highest abundance of species is STP1. The various plants showed differences regarding the most prevalent genera and species observed across the samples. For example, the most abundant genus was *Pseudomonas* in STP2 Influent, while it was *Acinetobacter* in STP12 Influent (Fig 4.10 A). With respect to relative abundances at the species level (Fig 4.10B), there is a clearer hierarchical clustering between influents and effluents. Various *Arcobacter* species have higher abundance influents than effluents. Conversely, various *Mycobacteria spp*. and *Mycolicibacteria spp*. were more abundant in effluent than influent. With respect to pathogen abundances, the influents and effluents cluster separately. Some ESKAPE pathogens (*E. faecium, K. pneumoniae, A. baumanii, P. aeruginosa* and *Enterobacter)* were more abundant in influents than effluents, whilst pathogenic *Mycobacteria spp.* and *Mycolicibacteria spp*. were more abundant in some effluents than influents.


STP12E1_S20
STP6E2_S11
STP5I_S9
STP1E_S2
STP2E_S4
STP11E2_S18
STP8I2_S14
STP12I2_S19
STP3E1_S6
STP1I2_S1
STP6I2_S10
STP7I2_S12
STP11I1_S17
STP2I_S3
STP3I_S5
STP7E2_S13
Collection Location
Pseudomonas
Acinetobacter
Aeromonas
Acidovorax
Comamonas
Cloacibacterium
Arcobacter
Thauera
Azoarcus
Dechloromonas
Hydrogenophaga
Flavobacterium
Paracoccus
Rhodobacter
Mycolicibacterium
Mycobacterium
Microbacterium
Streptomyces
Sphingomonas
Sphingobium
Novosphingobium
Porphyrobacter
Desulfovibrio
Klebsiella
Escherichia
Citrobacter
Enterobacter
Bacteroides
Bifidobacterium
Collinsella
Streptococcus
Romboutsia
Faecalibacterium
Blautia
Akkermansia
Mediterraneibacter
Methanobrevibacter
Prevotella
Shewanella
Acetoanaerobium
Aliarcobacter
Selenomonas
Megasphaera
Thiodictyon
Allochromatium
Marichromatium
Thiocystis
Thioflavicoccus
Thermochromatium
Limnohabitans
Polynucleobacter
Candidatus Methylopumilus
Fluviicola
Methylocystis
Aminobacter
Nakamurella
Dyella
Treponema
Empedobacter
Aurantimicrobium
Candidatus Planktophila
Rhodoluna
A
Showing NT.rpm for 10 taxa per sample on a symlog scale; Background: NID human CSF HC.
No filters applied.



Showing NT.rpm for 10 taxa per sample on a symlog scale; Background: NID human CSF HC.
No filters applied.



Showing NT.rpm for 10 taxa per sample on a symlog scale; Background: NID human CSF HC.
Taxa which are not present in the sample are represented by white cells. Taxa filtered out in this view have been hidden or grayed out. 1 filter(s) applied:
Pathogen Tags: known_pathogens

Figure 4.10: Bacterial diversity denoted by three heatmaps: at the genus level (A), species level (B) and the known pathogen diversity within the samples (C).

4.3 Discussion

4.3.1 Activities surrounding the WWTPs.

It is clear from the observational data that both wild and domestic animals frequented the sites for one important resource: water (Fig 4.2). The contribution of wildlife to ARG transfer has become more evident in the past 15 years. In the aquatic environment, ARG acquisition is typically attributed to the uptake of WW-polluted surface water (Abbassi *et al*., 2022). In this study, there was uptake of waters directly from WWTPs that had a high diversity of both ARGs (Fig 4.4) and bacteria (Fig 4.10) by both wild and domestic animals, further disseminating them into the local environment and the rural community with whom they closely live. WWTPs serve as both a source of water and food as well as reservoirs for pathogens and bacteria. In the current study, effluent samples showed reduced prevalence of ARGs and bacteria than the influent samples. Although the effluent water may not be consumed directly by the populations, zoonotic pathogens such as *C. botulinum* and *E. coli* may end up in water bodies used by the communities, posing a health threat upon exposure to them. Secure perimeter fencing may be an important facet in preventing the access to polluted waters by land animals, limiting the spread of pathogens and ARGs within the community. However, effluents, and effluent-impacted rivers (Table 4.1) are still used for agriculture (Gumbo *et al*., 2010) and likely wild animals. It may be worthwhile to perform long-term surveillance of ARGs in Vhembe to track their potential spread through these routes, although wild birds would be a challenge to deal with. This thus stresses the importance of regular maintenance and monitoring of WWTPs. High relative abundances of some ARGs in specific WWTPs where animals frequented were found in their effluents, some of which encode for multiple drug resistance such as *OXA-2, LCR* (β-lactamases), *erm35* and *opxB*, as well as those encoding for sulfonamides (*sulI, sulII, sulIII*) and tetracyclines (*tetO, tetW and tet-39*), which are commonly used for animal production and are persistent in rivers (Sabri *et al*., 2020a; Molechan *et al.* 2019).

Birds were one of the most abundant animals observed at the various WWTPs (Table 4.1), occurring at each plant and in high numbers. Their ability to fly allows them to cover much larger distances and thus distribute ARGs and ARBs. A wide variety of ARGs and resistant bacteria have been detected in many bird species globally (Abbassi *et al*., 2022). Some, such as the

Guinea fowl, are land birds, covering a relatively small area compared to flight birds such as egrets, geese, and storks. These migratory birds fly long distances and may carry ARGs and ARB with them. According to Abbassi *et al,* (2022), several factors contribute to the spread and persistence of ARGs within the wastewater environment: the presence of biofilms with diverse bacterial taxa, HGT by phages and discharge of fecal matter from wild animals into surface waters (rivers and lakes). At the Musina WWTPs, the bird populations seemed denser. The area is typically arid, and thus, this may explain the large number of birds on site in combination with land animals (elephants, cows, and sheep) as the plant served as an important water source. Near STP 6, further south of the region, a commercial chicken farm neighbored the WWTP; although chickens do not walk around, feathers and waste were being dumped at the plant, raising questions about possible contact with the indigenous wild birds. Antibiotic-resistant *Campylobacter* was found in wild Malaysian migratory birds near duck and chicken farms, whereby chicken isolates were found to have resistance against 12 antibiotics tested (Ibrahim *et al*., 2019). Transcriptome-based metagenomics of wild birds living at a WWTP in Australia showed high levels of antibiotic resistance genes within water bird species feeding at the plants, especially in ducks that fed by dabbing (Marcelino *et al.,* 2018). This indicates that wild birds may serve as vectors of ARGs from reservoir WWTPs; thus, including them in future studies may indicate the spread of ARGs within Vhembe, the country and perhaps globally, depending on the habits of the various bird species. Domestic animals co-grazing with wild animals have been found to share similar resistance genes against tetracyclines and sulfonamides (Van den Honert *et al.,* 2021).

Human aspects of the observations and questionnaire focused on workers' health and general good practices such as hand washing and eating on the plant. The AWARE study, conducted by Berglund and colleagues (2023), revealed that WWTP worker ARG abundance did not differ greatly from controls. However, women showed a greater abundance of disinfectant resistance genes than men, and the overall abundance differed by region (Romania *vs* Netherlands). Some plants in this study had predominantly female workers. Workers at plants are still at potential risk of symptomatic/asymptomatic infection by enteric, respiratory, and opportunistic pathogens. Thus, good practice and hygiene within plants remain important to not contribute to asymptomatic infections within a community (Poopedi *et al*., 2023). All workers reported handwashing before meals and wearing safety equipment at the plants, with only one worker reporting being sick with flu symptoms within two weeks before sample collection. Further studies in Vhembe may focus on this relationship between workers and WWTP to identify the risk in rural settings, elucidating gaps in the relationship between WWTPs, workers and the community.

4.3.2 ARG and Bacterial diversity

Some of the most abundant ARGs across the treatment plants were *sulI (A class 1 intergron encoding* sulfonamide resistance), *msrE* (encodes for erythromycin and streptogramin B) and genes encoding for tetracycline resistance (*tetO* and *tet-39*) (Fig 4.5). Antibiotic resistance genes *sulI* and *msrE* were also detected in all 16 samples (Fig 4.4), and *tetO* and *tet-39* were detected in all influent samples. This indicates their presence in the community, however, *tetO* mapped reads were reduced between effluent and influents of all WWTPs and *tet-39* was reduced in all plants but STP 3 (Table 4.6), as it was more prevalent in the influent samples. *tetO* has been detected in *Campylobacter* in two rivers in the Kwa-Zulu Natal Province of South Africa, influenced by human activities (Molechan *et al.,* 2019).

The ARGs *sulI, sulII* and *sulIII* are sulfonamide resistance genes commonly associated with class 1 integrons, small non-conjugative plasmids, and plasmids, respectively. They are found in gram-negative and gram-positive bacteria from animals, humans, river water, sediment, and wastewater (Nandi *et al*., 2004; Antunes *et al.,* 2005; Sabri *et al.*; 2020a; Sabri *et al.*, 2020b). *SulI* and sulfonamide antibiotics have been detected in a study conducted by Sabri and colleagues (2020b) in the Netherlands up to 20 km from a WWTP using qPCR, showing an increase downstream from the WWTP. They also indicated that *sulI* decreased in concentration during winter. The samples in the current work were all collected during summer, which may explain the high prevalence. However, factors such as physicochemical parameters that influence ARG transmission, such as suspended solids, were not determined. Interestingly *sulI* was more prevalent in effluent than influent in 4 plants (Table 4.5), indicating that WWTPs may concentrate this ARG, this may be due to its hardiness in the WWTP environment, treatment technology or the condition of the treatment plants (Sabri *et al.,* 2020a; Sabri *et al.,* 2020b; DWS, 2023).

The ARG *msrE* is a plasmid-borne gene, showing a reduction between influents and effluents in all plants except for STP3 (Table 4.5).and was also one of the most abundant genes in a study by Ekwanzala and colleagues (2020), along with other mobile genes such as *bla*$_{CTX-M}$, *bla*$_{OXA}$ and *sulI*. The core ARG matrix of 36 ARGs within the influent samples in the current study was the same number as in Ekwanzala's study (2020). However, the notable difference was the composition of those ARGs. In this study, genes encoding for antibiotic inactivation predominated, although some genes encoding for efflux pumps (mostly tetracyclines) were detected, such as *tetA, tetC, tetB-P, tetG tet-39*. Other ARGs in this study encoded efflux pumps capable of conveying resistance to multiple antibiotics such as *cmlA, oqxB.* Animals drinking from WWTPs may thus obtain antibiotic resistance from the wastewaters and be preemptively resistant to antibiotics used on them, or. they could seed the plants via fecal pollution or bacteria in their saliva. When observing the core resistome (Fig 4.7, 4.8), *carA* was commonly detected in all effluent samples but not influent ones. This ARG conveys macrolide resistance and forms part of the ABC-F subfamily proteins.

A question that arises is where the high prevalence of sulfonamide and tetracycline resistance in the influents came from. The people of the Vhembe District use water sources polluted by enteric pathogens (Traore et al., 2016; Potgieter *et al.,* 2020). Antibiotic resistance has been found in households in rural settings within Vhembe, but with higher resistance against macrolides, chloramphenicol, and β-lactams versus tetracyclines (Rakhalaru *et al*., 2023). This influence from raw sewage may be from runoff of abattoirs and agricultural activities in the area, such as cattle, pigs and chickens supplying wastewater to the plants (Bester *et al*., 2008; Tanih *et al*., 2015; Abia *et al.,* 2023). However, there is a limited understanding of the influence of agricultural, abattoir and veterinary wastewaters on the WWTPs in South Africa and Vhembe. Other indicators of animal influence, such as the presence of *Enterobacter cloaceae* were high in influent samples, but also detected in effluents (Fig 4.10C). It has been found within poultry farms with resistance to sulfonamides, tetracyclines and β-lactamases (Nandi and Hussain, 2013). Bacteria make up the majority of zoonotic diseases, with some known zoonotic bacteria such as *Arcobacter cryaerophillus, A. bultzeri* (animal hosts: sheep, chickens, cattle and pigs) and *Bordetella bronchiseptica* (cats and dogs) (Rahman *et al.,* 2020). *A. bultzer*i has been found in wastewater across selected WWTPs; it is reported as an emerging threat and has been showing an increase in antibiotic resistance (Ferreira *et al*., 2016).

One site, STP3, was an interesting case. Located on the border between Zimbabwe and South Africa along with STP2 and STP1, where many immigrants pass through, potentially introducing new ARGs not found in other areas. *SulI, tet-39* and *mrsE* were more prevalent in the effluent versus influent samples at this plant. Their hardiness may allow their dissemination through rivers via the effluents, which is a potential risk for those using effluent-impacted waters for agricultural activities and domestic use. STP 3 effluent also contained the highest number ARGs and unique genes, which were only detected in that sample. It had a tertiary treatment step for chlorination before release; however, the chlorination and some other parts of the treatment process were not properly functional at the time, like other plants in the Vhembe regio. Lamba and Ammahd (2017) found a 10-fold higher reduction of ARGs and integrons in secondary treatments coupled with UV and ozone versus chlorination. Chlorination can effectively remove ARB, but not ARGs when compared to other tertiary treatments. It is important to note that the tertiary treatments in the plants were either partially functional or dysfunctional, and plants in the Musina area were scheduled for maintenance shortly after sampling.

Another interesting site was STP11. It is a shutdown plant still receiving sewage from an indiscernible origin apart from perhaps some households nearby. It had a diverse group of birds at the edges and around the ponds, showing signs of domestic animals and local community members passing by the WWTP (Fig 4.3). The system was in an' overgrown state, like wetlands. The total reads decreased from 5249-324 mapped reads (93.8% reduction), and detected ARGs decreased from 53-30 from influent to effluent. The samples also appear visibly less turbid. This shows the potential of application of wetland plants, such as *Cyperus sexangualris* and *phragmites mauritianum*, which are both used traditionally in the region for activities such as flute making and construction, providing useful fibres (Dalu *et al.,* 2020; Magwede *et al.,* 2019). Closely related indigenous species such as *Phragmites australis, Cyperus prolifer* and *Cyperus papyrus*, as well as microalgae (*Tetradesmus obliquus* and *Tetradesmus reginae*), have been tested in constructed wetland microbial fuel cells for simultaneous production of electricity and wastewater treatment and found to possess adequate wastewater treatment and some energy production capabilities (Oodally *et al.,* 2019; Bolton and Randall, 2019; Ahiahanu *et al.,* 2022a). The algae present in the plants (Fig 4.2) could also perhaps be investigated, as indigenous microalgae have shown capabilities of producing electricity, treating wastewater, producing biofuels, sequestering carbon, and containing fatty acids of nutraceutical value (Ahiahonu *et al.,* 2022a; Ahiahonu *et al.,* 2022b;

Ahiahonu *et al.,* 2021). Constructed wetlands and microalgal wastewater treatment have been shown to significantly decrease antibiotics and ARGs such as *sulI, sulII tetO* and *intl1* (Chen *et al.,* 2015; Ovis-Sánchez *et al.,* 2023). This demonstrates the further need for maintenance campaigns and the development of strategies to mitigate the leaching of unique ARGs in the area, which may cause "outbreaks" of previously non-prevalent ARGs. A future focus of maintenance plans may be to incorporate low-maintenance, sustainable systems after treatment to buffer against ARG and bacterial dissemination.

In this study, a diverse bacteriome was revealed across selected plants in Vhembe, predominated by members from genera *Arcobacter, Aeromonas, Pseudomonas* and *Acinetobacte*r. Notable pathogens found in the influent samples predominantly cause gastroenteritis and others, capable of causing pneumonia, such as *Klebsiella pneumoniae* and *Pseudomonas aeruginosa,* were also detected. This may indicate a high degree of these infections within the population. *A. caviae* was the dominant pathogen within the detected samples (Fig4.11C) and has been found in extra-intestinal infections in China with resistance to tetracyclines (Song *et al*., 2023). *Aeromonas* spp. and *Pseudomonas* spp. have been recently found to possess high phenotypic resistance to tetracyclines, sulfonamides and β-lactamases in South African wastewaters (Govender *et al*., 2021).

This study also uncovered a notable prevalence of *Mycobacteriaceae* (*Actinobacteria*) from influent and effluent samples in four plants and STP5's influent. This indicates their presence in the community, their concentration in WWTPs, and their dissemination into the rural river systems. According to Akram and Attia (2023), some species, such as *M. avium* and *M. intracellulare* are non-tuberculosis mycobacteria (NTM), forming the *Mycobacterium Avium* complex and have been found in various matrices including soil, water, animals, and birds and poses a threat to vulnerable individuals such as those with HIV. They further note that typical treatments for these bacteria involved macrolide, fluoroquinolone, linezolid, clofazimine, rifampin, ethambutol, rifabutin, and aminoglycosides. Genes encoding for macrolide and aminoglycoside resistance formed part of the core resistome of this study. This may help inform about preemptive antibiotic resistance screening in healthcare settings where NTM may be prevalent in communities. In recent literature, information on the NTM, *M. xenopi,* infections is scarce, with a recent review focusing on extrapulmonary infections in European countries, USA, Canada, and New Zealand but not Africa (Rodari *et al.,* 2020). NTM are a diverse group of environmental pathogens; new species have been detected due to more effective culturing and detection techniques and an increase in susceptibility to NTM infection due to HIV. Furthermore, they are resistant to treatment due to their hydrophobic lipid-rich outer layer, which aids them in attaching to surfaces, and show higher resistance than other bacteria such as *E. coli*, and *Pseudomonas aeruginosa* (Falkinham *et al.,* 2015). Makuwa and colleagues (2023) detected high abundances of Actinobacter alongside other bacterial classes in a WWTP in the North-West Provice of South Africa. The resistance mechanisms of these bacteria could be more closely studied to understand their accumulation in WWTPs in Vhembe.

Identifying and categorizing the various bacteria (Fig 4.10) and ARGs (Fig 4.5) across entire communities within Vhembe has provided major insights into what is circulating. The presence of pathogenic bacteria and ARGs in effluent samples suggests the potential resistance and pathogens which may affect the community, including its animals, which rely on rivers influenced by these contaminants. Thus, approaches such as One Health may help in filling gaps in knowledge that have now arisen, such as where predominating ARGs and bacteria come from in WWTP systems and how they transfer between community members, animals (both wild and domestic) and the environment.

Chapter 5: Conclusion and Recommendation

5.1 Conclusion

This study aimed to contribute to this understanding of antibiotic resistance genes and associated bacteria within Vhembe, Limpopo, South Africa, using a metagenomics approach. Utilization of DNA sequencing technologies, such as next-generation sequencing (NGS), uncovered a broad spectrum of both ARGs and bacterial pathogens unmatched by previous studies. The findings of this study reveal the hardiness of some ARG and bacteria to wastewater treatment and the inefficiency of treatment by dilapidated WWTPs. Predominating ARGs across WWTP encode for tetracycline, macrolide, streptogramin B and sulfonamide resistance. Others which encode resistance against β-lactams and multiple drugs been detected using NGS. A broad host of bacteria in effluents and influents was detected, such as *Acinetobacteria, Aeromonas Pseudomonas, E. coli* and *Arcobacter,* with a notable accumulation of *Mycobactericae* in some effluents. Although some ARGs and bacteria were reduced, inadequate treatment by WWTPs in a critical state needs to be addressed. It is important to acknowledge that the data presented in this study does not elucidate dissemination by vectors and rivers, nor are the resistances phenotypically proven. Further research is highly recommended to provide a comprehensive insight on pathogen diversity in WWTPs, interactions with the animals and community members and on how ARGs and pathogens transfer within the water environment.

5.2 Study Limitations

- Not all treatment plants could be sampled, nor could both influents and effluents be sampled from all plants in Vhembe.
- Live bacteria and phenotypic expression of ARGs were not investigated.

5.3 Recommendation

- Composite samples may have been more favorable to account for fluctuations in wastewater composition throughout the day.

- It may be important to investigate the influence of surrounding animals on the WWTPs and *vice versa,* as well as conduct studies on wastewater from animal production facilities and veterinary clinics.
- Further investigations on the dissemination of ARGs may be important to look at from a One Health perspective to elucidate transmissions and origins of bacteria and ARGs.
- Wastewater treatment plants could be encouraged to assist in further surveys, as plants along the borders prove a prime target for wastewater-based epidemiology.

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Appendix A

Form used for data collection and observations at WWTPs and DNA data analysis of samples used for this study (Table 1A)

<u>WASTEWATER TREATMENT PLANT SURVEY.</u>

NB: Please administer the concent form first; if the respondent agrees to participate, you may proceed with the questionnaire.

Date of Visit: D/MM/YYYY) Treatment plant ID: G: SW1)

Treatment plant: GPS location:

NB: Where applicable, please tick (☑) in a box.

1. GEOGRAPHICAL INFORMATION

A. How many settlements does the WWTP receive wastewater from?

B. How can the majority (more than half) of settlements be described?

A. Urban ☐ B. Peri-Urban ☐ C. Rural

C. What is the closest settlement?

D. How many Hospitals does the WWTP get wastewaterwater from?

E. Which hospital is the nearest to the treatment plant

F. Which river does the WWTP effluent exit into?

G. Does the Treatment plant receive runoff from any commercial farms?

a. Yes ☐ b. No ☐

H. If Yes. Tick the appropriate farming products which are produced

a. Plant crops ☐ b. Cows ☐ c. Sheep ☐ d. Goats ☐ e. Chickens ☐ f. Pigs ☐ d. Other (please specify) ……………………………………….

b. What animals/insects (if any) are typically observed around the plant?

…………………………………………………….

2. INFRASTRUCTURE

A. What is the primary wastewater treatment technology used?

B. What is the secondary treatment technology used?

C. What is the tertiary treatment technology used?

D. What is the current flow rate?

…………………………………………………….

E. Has any failing equipment been reported in the past 2 weeks?

a. Yes ☐ b. No ☐

F. Does it have a direct influence on treatment performance?

a. Yes ☐ b. No

3. HYGIENE AND SANITATION

A. Is Handwashing mandatory prior to workers taking lunch?

a. Yes ☐ b. No ☐

B. Do employees eat in or around the facility?

a. Yes ☐ b. No ☐

C. Do the workers have a dedicated place to eat food like a mess hall/cafeteria?

a. Yes ☐ b. No ☐

D. What personal protective equipment are employees inside the plant mandated to wear?

a. Overalls ☐ b. Dust jackets ☐ c. Goggles ☐ d. Gloves ☐ e. Masks ☐ f. Other ☐ (please specify)…………………………………………

E. Has any employee reported being sick in the past 2 weeks?

a. Yes ☐ b. No ☐

F. If yes. What symptoms did they report:

a. Stomach (Nausea, vomiting, diarrhoea) ☐ b. Respiratory ☐ c. Urinary. d other ☐ (please specify)……………………………………

If there were specific diseases (ie. *E.coli* or Norovirus infection) reported, please specify below:

Are regular vaccinations mandatory for workers?

a. Yes ☐ b. No ☐

Table 1A: Nanodrop analysis of DNA extracts from Influent and effluent WW samples for sequencing

Sample ID	Quantity (ng/µL)	A260/280	A260/230
STP1I2	11.40	1.64	0.35
STP1E	31.20	1.98	1.39
STP2I	11.60	2.19	0.77
STP2E	32.30	2.01	1.38
STP3I	12.00	2.11	0.51
STP3E1	11.60	2.38	0.21
STP5I	62.50	1.32	0.57
STP6I2	27.10	1.85	1.30
STP6E2	24.00	1.85	0.29
STP7I2	37.30	1.76	0.57
STP7E2	12.50	1.67	0.56
STP8I2	12.20	1.85	0.62
STP11I1	26.40	2.02	0.77
STP11E2	16.40	1.33	0.42
STP12I2	52.40	1.94	1.61
STP12E1	47.80	1.91	1.46