# The Prevalence of Selected Emerging Pathogenic Species in Wastewater and Receiving Water Bodies

**Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy (PhD): Biotechnology in the Department of Biotechnology and Food Science, Faculty of Applied Sciences, Durban University of Technology, Durban, South Africa**


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# DECLARATION

I, Ms. R Govender (Student number – 19500281) and Prof TA Stenström do hereby declare that in respect of the following dissertation:

Title: **The Prevalence of Selected Emerging Pathogenic Species in Wastewater and Receiving Water Bodies**

**1.** As far as we ascertain:

a) no other similar dissertation exists.

**2.** All references as detailed in the dissertation are complete in terms of all personal communication engaged in and published works consulted.

16 March 2022

**Signature of student** **Date**

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# AUTHOR'S DECLARATION

This study presents original work by the author. It has not been submitted in any form to another academic institution. Where use was made of the work of others, it has been duly acknowledged in the text. The research described in this dissertation was carried out in the Department of Biotechnology and Food Technology, Faculty of Applied Sciences, Durban University of Technology, South Africa, under the supervision of **Prof TA Stenström, Prof SKK Pillai, Prof FM Swalaha** and **Prof F Bux**.

STUDENT'S SIGNATURE

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# ABBREVIATIONS

| | |
|---|---|
| Acb | : *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex |
| ARG | : Antibiotic Resistance Genes |
| APHA | : American Public Health Association |
| ATCC | : American Type Culture Collection |
| AST | : Antibiotic Susceptibility Testing |
| BacLight kit | : LIVE/DEAD® BacLight™ Bacterial Viability Kit |
| bp | : Base Pair |
| CDC | : Centers for Disease Control and Prevention |
| CFU | : Colony Forming Units |
| CFU/g | : Colony Forming Units per gram |
| CFU/mL | : Colony Forming Units per millilitre |
| CLSI | : Clinical and Laboratory Standards Institute |
| COVID-19 | : Corona Virus Disease of 2019 |
| ddPCR | : droplet digital Polymerase Chain Reaction |
| DNase | : Deoxyribonuclease |
| D/S | : Downstream |
| DSF | : Diffusible signal factor |
| ESBL | : Extended Spectrum *β*-Lactamase |
| ESKAPE | : *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* species |
| FCM | : Flow cytometry |
| FIDSSA | : Federation of Infectious Diseases Societies of Southern Africa |
| FISH | : Fluorescent *in situ* Hybridization |
| Flow-FISH | : Flow cytometry Fluorescent *in situ* Hybridization |
| FSC | : Forward Scatter |
| gDNA | : Genomic Deoxyribonucleic acid |
| GC | : Genomic Copies |
| GC/mL | : Genomic copies per millilitre |
| h | : Hours |

| | |
|---|---|
| HIV | : Human Immunodeficiency Virus |
| IVD | : *In Vitro* Diagnosis |
| ISO | : International Organization for Standardization |
| LOD | : Limit of Detection |
| LPS | : Lipopolysaccharide |
| MALDI-TOF MS | : Matrix-Assisted Laser Desorption Ionisation-Time of Flight Mass Spectrometry |
| MAR | : Multiple Antibiotic Resistance |
| MARI | : Multiple Antibiotic Resistance Index |
| MDR | : Multiple Drug Resistance |
| min | : minutes |
| mL | : millilitre |
| n | : Sample number |
| NCBI | : National Centre for Biotechnology Information |
| NDoH | : National Department of Health |
| ng | : Nanogram |
| nm | : Nanometres |
| NTC | : No Template Control |
| NWWTP | : Northern Works Wastewater Treatment Plant |
| OR | : Odds Ratio |
| PBS | : Phosphate Buffered Saline |
| PCR | : Polymerase Chain Reaction |
| PI | : Propidium Iodide |
| PMA | : Propidium Monoazide |
| PMT | : Photo Multiplier Tube |
| PTC | : Positive Template Control |
| qPCR | : Quantitative Polymerase Chain Reaction |
| RNase | : Ribonuclease |
| RP | : Phenotypic Antibiotic Resistance |
| SA | : South Africa |
| SAASP | : South African Antibiotic Stewardship Programme |
| SARAMIS | : Spectral Archiving and Microbial Identification System |
| SSC | : Side Scatter |

| | |
|---|---|
| Stats SA | : Statistics South Africa |
| TAE | : Tris- acetate EDTA |
| TE | : Tris-EDTA |
| TTSS | : Type III secretion system |
| UNICEF | : United Nations Children's Fund |
| U/S | : Upstream |
| UTI | : Urinary Tract Infection |
| VBNC | : Viable but Not Culturable |
| VG | : Virulence Genes |
| WHO | : World Health Organization |
| WWTP | : Wastewater Treatment Plant |
| WWTPs | : Wastewater Treatment Plants |
| $\chi^2$ | : Chi squared valued |

# CONFERENCES/PAPERS

## CONFERENCE PRESENTATION

SASM Conference, Muldersdrift, Johannesburg, South Africa, 4-7 April 2018. "Species Diversities, Antibiotic Profiling and Virulence Signatures of *Aeromonas* and *Pseudomonas* in Wastewater Treatment Plants and Recipient Surface Water". Govender, R; Adegoke, A. A, Singh, G, Pillai, SKK, Faizal Bux, F, Stenström, T. A.

## PAPERS PUBLISHED

**See Appendix A (The papers are part of Chapter 4 and Chapter 5 of the thesis)**

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

**Govender, R**., Amoah, I. D., Kumari, S., Bux, F. and Stenström, T. A. 2020. Detection of multidrug resistant environmental isolates of *Acinetobacter* and *Stenotrophomonas maltophilia*: a possible threat for community acquired infections? *Journal of Environmental Science and Health, Part A*: 1-13.

# TABLE OF CONTENTS

# LIST OF FIGURES

# LIST OF TABLES

# ABSTRACT

Antibiotic resistance is one of the biggest threats to global health, due to the excessive use of antibiotics, among other factors. Aquatic environments are considered hotspots for antibiotic-resistant bacteria and genes due to pollution caused by various anthropogenic activities. In this study, four emerging opportunistic pathogens viz., *Acinetobacter* spp., *Pseudomonas* spp., *Aeromonas* spp., and *Stenotrophomonas maltophilia* were investigated to understand their distribution, source, and resistance patterns in wastewater and surface water. Among these, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* have been listed by the World Health Organization (WHO) in 2017 as priority bacteria for further research and development. This study focused on the Umhlangane River, located in the north of Durban, in KwaZulu Natal, South Africa. The possible effect of anthropogenic activities such as discharges from wastewater treatment plants (WWTPs), hospitals, informal settlements, and veterinary clinics on the occurrence of antibiotic-resistance, and virulence signatures of the targeted organisms, was investigated. Sixty samples (12 wastewater, 48 surface water) were collected monthly (November 2016 to April 2017). This included influent and effluent of a wastewater treatment plant (WWTP) and four additional sampling sites (upstream and downstream of the WWTP, a hospital, an informal settlement, and a veterinary clinic). In addition, to the sixty samples, further samplings of aquatic plants ($n$=16) and sediments ($n$=16) were done in October 2017, specifically for the isolation of *Stenotrophomonas maltophilia*. The isolation and enumeration were carried out on selective media for each bacterium. The PCR positive isolates were identified using Matrix-Assisted Laser Desorption Ionization -Time of Flight Mass Spectrometry (MALDI-TOF MS) and 16S rRNA sequencing. In addition, advanced methods such as Flow Cytometry (FCM) and Droplet Digital PCR (ddPCR) were used to detect and quantify the bacteria, in comparison to conventional methods. The multiple antibiotic resistance (MAR) index was calculated to ascertain the contribution of these pollution sources to the proliferation of antibiotic-resistant bacteria in surface water. Varying counts ($\log_{10}$ CFU/mL) of *Aeromonas* spp. (2.5±0.8 to 3.3±0.4), *Pseudomonas* spp. (0.6±1.0 to 1.8±1.0) and *Acinetobacter* spp. (2.0±1.5 to 2.6±1.2) were obtained. *S. maltophilia* was found in the water column only at two sites and ranged from 2.7±0.3 to 4.1±1.0 $\log_{10}$ CFU/mL. However, it was found abundantly in the plant rhizosphere (3.6±0.1 to 4.2±0.6 $\log_{10}$ CFU/mL) and sediment (3.8±0.1 to 5.0±0.1 $\log_{10}$ CFU/mL) samples. The major *Aeromonas* species identified by MALDI-TOF MS was *A. hydrophila / caviae* (58%) whilst *P. putida* (51%) was common amongst the *Pseudomonas*

isolates. The *Acinetobacter* genus was dominated by the *Acinetobacter baumannii* complex (26%), in contrast, all *Stenotrophomonas maltophilia* identities were confirmed via Polymerase Chain Reaction (PCR) and MALDI-TOF MS. *Aeromonas* (71%) and *Pseudomonas* (94%) isolates displayed resistance to three or more antibiotics. *Aeromonas* isolates displayed high resistance against ampicillin and had higher MAR indices, downstream of the hospital. The virulence gene, *aer* in *Aeromonas* was positively associated with the antibiotic resistance gene $bla_{OXA}$ ($\chi^2$=6.657, $p$<0.05) and the antibiotic ceftazidime ($\chi^2$=7.537, $p$<0.05). *Pseudomonas* exhibited high resistance against third-generation cephalosporins in comparison to carbapenems. Some *Pseudomonas* and *Aeromonas* isolates were extended-spectrum $\beta$-lactamase producing bacteria as the $bla_{TEM}$ gene was detected in *Aeromonas* spp. (33%) and *Pseudomonas* spp. (22%). All *S. maltophilia* isolates were resistant to the antibiotic's trimethoprim-sulphamethoxazole, meropenem, imipenem, ampicillin, and cefixime. *Acinetobacter* isolates were resistant to trimethoprim-sulphamethoxazole (96%) and polymyxin (86%). The genes coding for resistance against these antibiotics were detected in both *S. maltophilia* and *Acinetobacter*. Efflux pump genes were detected in all isolates of *S. maltophilia*. High MAR indices were observed in isolates of *Pseudomonas, S. maltophilia*, and *Acinetobacter* at the hospital site. However, *Aeromonas* spp. had the highest MAR in isolates from the WWTP effluents. A comparative analysis of three different methods was performed to understand their applicability and accuracy in detecting these pathogens from wastewater samples. The total viable count using the LIVE/DEAD Baclight bacterial viability kit measured an average count ($\log_{10}$ bacteria per mL) of 7.8±0.03 (influent) and 6.7±0.07 (effluent) using the Flow Cytometer. The total viable count using the BacLight kit was higher than the total plate count, which was 6.46±0.02 and 4.63±0.07 $\log_{10}$ CFU/mL for influent and effluent, respectively. Similarly, the concentration for each of the target bacteria determined using Flow Cytometry combined with Fluorescent-*In situ* hybridization (Flow-FISH) method ranged from 5.41±0.07 to 5.92±0.02 (influent) and 3.43±0.2 to 4.31±0.15 (effluent) $\log_{10}$ bacteria per mL which was higher than the selective plate counts (3.81±0.35 to 4.17±0.1 and 3.16±0.17 to 3.7±0.20 $\log_{10}$ CFU/mL, for influent and effluent respectively). The ddPCR results obtained showed the highest concentration of bacteria from both influent and effluent samples in comparison to the Flow-FISH and the plate count methods, indicating the sensitivity of this method in detecting both live and dead cells. *Pseudomonas* was observed to be dominant and was found in the concentration of 7.19±0.24 copies per mL (influent) and 6.48±0.20 copies per mL (effluent) while *S. maltophilia* (influent: 5.4 ± 0.90 copies per mL effluent: 4.53±0.57

copies per mL) was detected in the lowest concentration. A similar trend was observed in comparison to the data from the plate counts, albeit at lower concentrations. This study, therefore, makes significant contributions in several areas; firstly, it shows the abundance of opportunistic, antibiotic-resistant, and virulent bacteria in wastewater and surface water within Durban. It further demonstrates that these bacteria are mainly from anthropogenic sources such as hospitals and WWTPs. Additionally, the findings indicate the potential for community-acquired infections with these bacteria, necessitating the need for risk reduction interventions aimed at reducing environmental pollution and exposure.

# Chapter 1: Introduction

Water is essential for life and is considered a fundamental human right. With the growing demand and the depleting supplies, water is seasonally becoming a scarce natural resource locally and globally (Crookes, Hedden, and Donnenfeld 2018). The major factors contributing to the increasing water crisis in South Africa include insufficient maintenance of water infrastructure, frequent droughts as a result of climate change, unequal access to water and sanitation, the degeneration of water quality, and the shortage of experienced water engineers (Department of Water and Sanitation 2018). These different factors impact the economy of the country and more importantly, it affects the wellbeing of every individual in South Africa. Several rural communities and informal settlements in South Africa still do not have proper access to water services. These communities rely on natural water sources such as rivers, streams, and dams for daily activities; thus, they are at a higher risk, by using unprotected water sources (Tissington *et al.* 2008). This is further highlighted by the general household survey conducted by Statistics South Africa (2017), which stated that 88.6% of South African households had access to piped water. Though access to water is progressing, 3.7% of households still rely on water from rivers, dams, streams, wells, and stagnant water pools for their daily needs (Statistics South Africa 2017). As a result, many people are at risk of contracting various waterborne diseases such as diarrhoea, which is a major concern since these diseases are responsible for a large percentage of morbidity and mortality globally, especially in children under five [World Health Organisation (WHO) and United Nations Children's Fund (UNICEF) 2013].

Surface water contamination arises from various anthropogenic activities such as discharge of treated and untreated sewage from malfunctioning treatment plants, runoff from urban, commercial, and agricultural land. Discharge of stormwater runoff via stormwater drains that run into surface water is also responsible for pathogen contamination (Pandey *et al.* 2014). In addition to the pathogens that are excreted by humans and animals, some may occur as natural commensals in the environment. There are various opportunistic pathogens that one can come into contact with while using contaminated water. Some of those are the focus of this study which includes *Aeromonas* spp., *Pseudomonas* spp., *Acinetobacter* spp., and *Stenotrophomonas maltophilia*.

The emerging pathogens which are widely occurring in aquatic environments are mainly responsible for causing nosocomial infections, which are healthcare setting-associated infections (Looney, Narita and Mühlemann 2009; Howard *et al.* 2012; Khan, Ahmad and Mehboob 2015; Batra, Mathur and Misra 2016). The emergence of opportunistic and nosocomial bacterial infections due to multiple-drug resistant (MDR) non-fermentative Gram-negative strains, has become an important impediment in recent years (Agarwal et al. 2017). These infections extend hospital stays, increase the cost of treatment, and may even lead to mortality (Ginawi *et al.* 2014).

A common manifestation of an *Aeromonas* infection is diarrheal disease (Cortés-Sánchez *et al*. 2019). Aeromonads are well known as enteric pathogens as they have been associated with many food and waterborne outbreaks. They have been isolated from people with traveller's diarrhoea and have been associated with diseases such as gastroenteritis, septicaemia, skin diseases, soft tissue, and muscle infections (Igbinosa *et al.* 2012a). *Acinetobacter* is associated with infections related to wounds, the gastrointestinal and respiratory tracts. It is found inhabiting oral biofilms and if aspirated into the lower respiratory tract there is a likelihood of developing pneumonia (Kanafani and Kanj 2013). *Pseudomonas* has been reported to be responsible for bacteraemia, ventilator-associated pneumonia, urinary tract infections, skin and soft-tissue infections (Bassetti *et al.* 2018), while *S. maltophilia* has been responsible for severe nosocomial bloodstream infections and pneumonia (Gozel, Celik and Elaldi 2015).

*Acinetobacter* and *Pseudomonas* are part of the highest category of a WHO published list of priority pathogens. This list comprises of 12 families of antibiotic-resistant bacteria that are classified as a concern and a threat to human health (World Health Organisation 2017). The three categories viz., critical, high, and medium, are based on the urgency of the need for the development of novel antibiotics. These listed bacteria are resistant to many antibiotics including carbapenems and third-generation cephalosporins, which are supposedly the most effective antibiotics available. Some of the selected pathogens in this study are categorized as ESKAPE *(**E**nterococcus faecium, **S**taphylococcus aureus, **K**lebsiella pneumoniae, **A**cinetobacter, **P**seudomonas aeruginosa, **E**nterobacter)* pathogens, as they can avoid the action of antibiotics and display new paradigms in the transmission, pathogenesis, and resistance of infectious disease. Therefore, they are a concern for water treatment facilities.

Even though the transmission of pathogens has been slowed down and their clinical manifestations counteracted by the widespread use of antimicrobial agents, there has still been an increase in antimicrobial resistance due to the selective pressure brought about due to the use of antibiotics (Beceiro, Tomás and Bou 2013). Antimicrobial resistance and virulence are impacted by each other therefore an understanding of their impact and how their regulation affects each other will enhance the specificity of the treatment (Schroeder, Brooks and Brooks 2017). However, information gaps about governance and surveillance platforms of antimicrobial resistance exist, especially in low to middle-income countries including South Africa (Wernli *et al.* 2017), which limits the attempts to control antimicrobial resistance. In South Africa, four serious outbreaks of antimicrobial resistance were reported at the national level. As a result, the National Department of Health published the South African Antimicrobial Resistance Strategy framework 2018-2024 (National Department of Health, 2015) to concentrate on antimicrobial resistance in the healthcare/clinical settings. However, there is an evident lack in the policy, concerning the containment and tracking of environmental isolates to prevent them from reaching healthcare settings, although the environmental isolates are considered as one of the main contributors to antimicrobial resistance (Ekwanzala *et al.* 2018). There are antibiotic awareness campaigns that are supported by The South African Antibiotic Stewardship Programme (SAASP) and the Federation of Infectious Diseases Societies of Southern Africa (FIDSSA) in association with the Department of Health, Forestry and Fisheries, and other professional organizations. These campaigns help prevent the misuse and abuse of antibiotics and improve the knowledge about antibiotic resistance.

As pointed out by Tissington *et al.* (2008) several rural communities in South Africa still do not have access to clean water and thus would often resort to using unprotected water sources for their daily needs. According to a survey conducted by Statistics South Africa, only 44.4% population had access to clean water inside their dwelling, while 30 percent of households had taps within their stand and the remaining made use of communal taps and natural water sources such as rivers and dams (Statistics South Africa, 2017). The water problems impact negatively on the health of local communities, resulting in exposed communities experiencing disease outbreaks from consuming untreated or contaminated water. The water crisis is already having a significant impact

on economic growth and the well-being of everyone in South Africa (Schreiner, Mungatana, and Baleta 2018). This has been further highlighted with the current Corona Virus Disease of 2019 (COVID-19) pandemic where the supply of water to rural areas is non-existent.

The Umhlangane River, selected for this study, is situated in Durban, KwaZulu-Natal, South Africa, and is used by the local communities for recreation, irrigation, and domestic purposes because of the unavailability of piped water. There is not much data published with reference to the selected pathogens that are part of this study. This study will provide information that can create public awareness about the current state of the water environment in relation to emerging opportunistic pathogens (that are on the WHO priority list), rapid and accurate detection methods, and hopefully encourage the implementation and action policies of antibiotic-resistant bacteria in the environment. This will be achieved by evaluating the presence and concentration of selected emerging pathogens in aquatic environments using conventional, molecular, and integrative approaches as well as to determine the role of WWTPs in the occurrence and spread of selected antibiotic-resistant bacteria to surface water.

## 1.1 Research Objectives

To achieve the aim of this study, it was divided into the following objectives:

1. To isolate, quantify, characterise and identify emerging pathogens belonging to *Acinetobacter*, *Pseudomonas*, and *Aeromonas* spp. and *Stenotrophomonas maltophilia* from surface water/wastewater.
2. To determine the antibiotic resistance/virulence gene profiles of the isolates through phenotypic and genotypic methods.
3. To quantify target organisms (*Acinetobacter* spp., *Aeromonas* spp., *Pseudomonas* spp. and *Stenotrophomonas maltophilia*) in wastewater (influent and effluent) using droplet digital polymerase chain reaction (ddPCR) and to determine the viability and total count of the target bacteria in the samples using Flow Cytometry (FCM) combined with Fluorescent *in situ* Hybridisation (FISH).

## 1.2 Thesis Structure

This thesis is divided into seven chapters. The introduction briefly describes the background of the study, highlighting the aims and objectives. Chapter two is a critical review of the literature, while chapter three describes some of the general methodologies. Chapter four covers the identification, total concentration, AR, and virulence gene profiling in *Aeromonas* and *Pseudomonas* species from wastewater and the surface water recipient. In chapter five, the presence of antibiotic-resistant *S. maltophilia* and *Acinetobacter* in the water matrices were discussed with reference to the possible threat of community-acquired infections from resistant isolates. Advanced detection methods are important to be able to prevent a public health problem in the event of an outbreak and in chapter six, the relatively new and advanced methods of flow cytometric analysis of viability and the combination of FCM with FISH for the detection/quantification of the target bacteria was investigated. ddPCR is another advanced method that was used for the quantification and detection of target bacteria in wastewater. Chapter seven is the general summary and conclusion of the research carried out.

# Chapter 2: Literature Review

## 2.1 Waterborne emerging pathogens

### 2.1.1 *Aeromonas* spp.

Aeromonads are Gram-negative, oxidase, catalase-positive rod-shaped, facultative anaerobic, non-spore forming bacteria that are widely distributed in the aquatic environment. This genus is comprised of two groups: the psychrophiles and the mesophiles. The mesophiles may be pathogenic to humans and consist of *Aeromonas hydrophila*, *Aeromonas caviae*, *Aeromonas veronii* subsp. *sobria*, *Aeromonas jandaei*, *Aeromonas veronii* subsp. *veronii* and *Aeromonas schubertii* (Obi *et al*. 2007). Aeromonads have been isolated from diverse aquatic sources such as surface water (Abulhamd 2010), bottled water, chlorinated (Latif-Eugenín *et al.* 2017), and unchlorinated water (Chauret *et al.* 2001; El-Taweel and Shaban 2001; Villari *et al.* 2003; Odeyemi and Ahmad 2017). *Aeromonas* can grow in biofilms in water distribution networks, and they have been found to develop resistance against chlorination (Igbinosa *et al.* 2012a). They have been found in the intestinal tract of humans and animals, raw sewage, treated effluents, surface water contaminated with sewage, and activated sludge (Goñi-Urriza *et al.* 2000a; Khajanchi *et al.* 2010). *Aeromonas* is becoming well known as enteric pathogens as they have been associated with different illnesses such as gastroenteritis (Janda and Abbott 2010) where they cause severe diarrheal disease in children, the elderly or immunocompromised (Igbinosa *et al.* 2012a), soft tissue, and wound infections (Rutteman *et al.* 2017) and more serious conditions such as septicaemia (Morinaga *et al.* 2011) and necrotizing fasciitis (Ren *et al.* 2017). The pathogenicity of *Aeromonas* depends on several factors and it is associated with the expression of genes that encode toxins (*exoA, Alt, Act* etc.), structural components (*flaA, maf-5, flp*), secretion systems, and proteins that are associated with metals (Tomás 2012; Fernandez-Bravo and Figueras, 2020). These virulence factors are discussed further in Chapter 2: Section 2.4. In South Africa, *Aeromonas* has been found in surface waters (Igbinosa *et al.* 2017), treated wastewater, and receiving surface water (Olaniran, Nzimande, and Mkize 2015). In the Limpopo Province in South Africa, *Aeromonas* was isolated from 13.3% of chronic diarrhoea HIV (Human Immunodeficiency Virus) patients (Obi and Bessong 2002).

Due to the ubiquitous nature of the genus *Aeromonas*, humans can easily encounter contaminated reservoirs of this bacteria, such as aquatic environments and become infected with pathogenic species of this bacteria. This is of concern for a country like South Africa with a high prevalence of HIV patients.

### 2.1.2 *Pseudomonas* spp.

*Pseudomonas* species are part of the category of emerging waterborne pathogens (Sharma, Sachdeva, and Virdi 2003). These ubiquitous Gram-negative, oxidase-positive, motile bacteria inhabit diverse environments such as soil, plants, sediments, and surface water (Noura *et al.* 2009). Their ability to proliferate and survive in poor physical and chemical conditions is due to their nutritional requirements being very meagre (Igbinosa, Igbinosa, and Okoh 2015). *Pseudomonas* species are non-spore-forming organisms that can produce pigments such as pyocyanine (green-blue) and pyorubin (yellow-green) (Murray *et al.* 1995). Among the different species of *Pseudomonas*, *Pseudomonas aeruginosa* is most commonly considered pathogenic; however, other important species of concern include *Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas stutzeri*, *Pseudomonas mendocina*, *Pseudomonas alcaligenes*, *Pseudomonas pseudoalcaligenes* and the *Pseudomonas* species CDC group (Igbinosa, Igbinosa and Okoh 2015). *Pseudomonas aeruginosa* is widely involved in nosocomial diseases (Pachori, Gothalwal and Gandhi 2019), and is difficult to treat when strains are MDR, which has been reported for more than 10% of *P. aeruginosa* strains isolated from patients in Europe (Azam and Khan 2019). *Pseudomonas* spp. have developed different resistance strategies: some mechanisms are intrinsic and based on efflux pumps, low permeability of the outer membrane, or antibiotic-inactivating enzymes encoded by chromosomal genes, allowing resistance against many antibiotic classes such as $\beta$-lactams, aminoglycosides, phenicol's, cyclins, macrolides, or quinolones (Li, Plésiat and Nikaido 2015; Azam and Khan 2019). This opportunistic pathogen causes diseases mainly in the immunocompromised group (Gellatly and Hancock 2013). They are responsible for skin infections, wound infections, chest infections, urinary tract infections, and bloodstream infections (Bassetti *et al.* 2018). Infections can result in fatalities such as septicaemia and pneumonia (Gauglitz, Shahrokhi, and Williams 2017). Various potential virulence factors allow for the colonization and infection of individuals. This includes bacterial cell surface virulence factors

(lipopolysaccharides, pili or fimbriae, flagella, alginate) and secreted virulence factors (protease enzymes, Exotoxin A, Phospholipase C, Pigments, Rhamnolipid, Lipase, Histamine, Exoenzyme S, Leukocidin). A common feature of *Pseudomonas* is the type III secretion systems that can inject toxins (*ExoY, ExoS, ExoT, and ExoU*) into the host cells (Igbinosa, Igbinosa, and Okoh 2015). Recent studies have shown that the prevalence of multiple antibiotic-resistant *Pseudomonas* strains is increasing, and few antibacterial agents are being developed that are effective against resistant *Pseudomonas* (Mhondoro *et al*. 2019). Currently, carbapenem-resistant *P. aeruginosa* is on the priority 1: critical category of the list of bacteria that need antibiotics to be developed to prevent death from infections that it is responsible for (WHO, 2017).

### 2.1.3 *Acinetobacter* spp.

*Acinetobacter* species are Gram-negative, oxidase-negative, non-motile Coccobacilli (Bergogne-Berezin and Towner 1996) which were present in wetlands, ponds, fish farms, and WWTPs (Al Atrouni *et al.* 2016). They have been isolated from healthcare facilities (Dijkshoorn, Nemec, and Seifert 2007) where they are thought to be responsible for nosocomial infections. The *Acinetobacter* genera are quite diverse, many of them are non-pathogenic organisms that are found in the environment (Wong *et al.* 2017). However, there are medically relevant infection-causing species such as *A. baumannii, A. calcoaceticus, A. pitti,* and *A. nosocomialis*. Together they are known as the *Acinetobacter calcoaceticus-Acinetobacter baumannii* (Acb) complex (Nemec *et al.* 2015; Cosgaya *et al.* 2016). This complex has been isolated from human tissues and healthcare facilities. Individuals with compromised immune systems and those with underlying conditions such as obstructive lung disease or diabetes mellitus are at risk for contracting infections (Chen *et al.* 2001). *Acinetobacter* spp. is responsible for urinary tract infections (Falagas *et al*. 2015), bacteraemia (Garnacho-Montero *et al*. 2015), and meningitis (Basri *et al.* 2015) as well as wound infections (Falagas *et al*. 2015). The most likely sources of transmission of these organisms in hospitals are via catheters (Rebic *et al.* 2018), water baths, and humidifiers (Wong *et al.* 2017) as well as from person to person (Joly-Guillou 2005). Other species that have been occasionally reported to cause mainly catheter related bloodstream infections include *Acinetobacter haemolyticus, Acinetobacter johnsonii, Acinetobacter junii, Acinetobacter nosocomialis, Acinetobacter pittii, Acinetobacter schindleri,* and *Acinetobacter ursingii* (Wisplinghoff *et al.*

2004; Higgins *et al.* 2010). Virulence factors that have been identified in *Acinetobacter* spp., includes the outer membrane porins, phospholipases, proteases, lipopolysaccharides (LPS), capsular polysaccharides, protein secretion systems, and iron-chelating systems (McConnell, Actis and Pachón 2013; Antunes, Visca and Towner, 2014; Lin and Lan 2014). Carbapenems (imipenem, meropenem, and doripenem) have generally been considered the antibiotics of choice to treat *A. baumannii* infections, due to their effective activity and their favourable safety (Doi, Murray and Peleg 2015). However, the increased resistance of *A. baumannii* to carbapenems has brought about the need to survey alternative therapeutic options (Doi, Murray, and Peleg 2015). Carbapenem-resistant *A. baumannii* strains are often resistant to other commonly used antibiotics. These strains remain susceptible to only a limited number of antibiotics, such as minocycline/tigecycline and polymyxins (colistin and polymyxin B) (Lin and Lan 2014; Doi, Murray and Peleg 2015). Therefore, the World Health Organization has listed carbapenem-resistant *A. baumannii* in the critical priority list of pathogens (WHO, 2017), thus highlighting them as an increasingly growing public health problem globally.

### 2.1.4 *Stenotrophomonas maltophilia*

*S. maltophilia* is an intrinsically MDR nosocomial pathogen and it is widely distributed in the environment (Gulcan, Kuzucu, and Durmaz 2004). It is a Gram-negative, non-fermentative, aerobic, motile organism that has emerged globally as an important opportunistic human pathogen (Brooke 2012; Wang, Wang, and Yang 2018). *S. maltophilia* is considered as the third most frequent nosocomial pathogen among non-fermentative bacteria, after *P. aeruginosa* and *Acinetobacter* spp. (Wang, Wang, and Yang 2018). *S. maltophilia* has been isolated from both clinical settings such as hospitals (Cervia, Ortolano and Canonica 2008) and from environmental sources such as wastewater (García-León *et al.* 2014), surface water sediments (Dungan, Yates and Frankenberger 2003), and plant roots (García-León *et al.* 2014). *S. maltophilia* is responsible for hospital and community-acquired infections in, incapacitated or immunocompromised patients through direct contact, ingestion, aspiration, aerosolization of potable water or the hands of healthcare workers (Guyot, Turton and Garner 2013) and was reported to have a mortality rate of 37.5% (Falagas *et al.* 2009). This bacterium is increasingly responsible for clinical illnesses such as pneumonia, sepsis, bacteraemia, septic arthritis,

meningitis, respiratory tract, and urinary tract infections (Looney, Narita and Mühlemann 2009; Sumida *et al.* 2015; Hu *et al.* 2016).

*S. maltophilia* has been identified as one of the leading MDR organisms in hospital settings (Bostanghadiri *et al.* 2019). This is due to their high levels of intrinsic and acquired resistance against a range of antibiotics which include fluoroquinolones and aminoglycosides (Alonso and Martinez 1997; Zhang, Li and Poole 2000) *β*-lactams, macrolides, fluoroquinolones, aminoglycosides, chloramphenicol, tetracyclines, polymyxins, and trimethoprim-sulfamethoxazole (Wu *et al.* 2006; Chang *et al.* 2007). Antimicrobial resistance mechanisms identified in *S. maltophilia* include the expression of antibiotic hydrolyzing/modifying enzymes, membrane permeability altering (Hu *et al.* 2008), and multidrug efflux systems (Huang, Somers, and Wong 2006). The intrinsic resistance of *S. maltophilia* is linked with low membrane permeability, efflux pumps, and the intrinsic beta-lactamases L1 and L2 (Sánchez, Alonso and Martinez 2002; Crossman *et al.* 2008; Mojica *et al.* 2019). *S. maltophilia* can acquire the following genes, dihydropteroate synthase (*sul1* and *sul2*), and dihydrofolate reductase (*drfA*) by horizontal gene transfer (Sánchez 2015) which are the key mechanisms of sulphamethoxazole resistance (Toleman *et al.* 2007).

*S. maltophilia* is known to exhibit its pathogenicity through:

(1) pili/flagella/fimbrial/adhesins; these contribute to adherence, auto-aggregation, and colonization of biotic and abiotic surfaces.

(2) outer membrane LPS is instrumental in biofilm formation and resistance against antibiotics as well as complement-mediated cell killing.

(3) diffusible signal factor (DSF) which is involved in quorum sensing (QS), which in turn facilitates motility, extracellular enzymes production, LPS synthesis, microcolony formation, and antibiotic and heavy metal ion tolerance; and

(4) extracellular enzyme production (proteases, lipases, esterase, DNase, RNase, and fibrinolysin) (Looney 2005; Abbott *et al.* 2011; Brooke 2012).

## 2.2 Potential sources of emerging bacterial pathogens in surface water

The aquatic environment is an important source and route for the dissemination of antibiotic-resistant bacteria (Martínez 2008; Bengtsson-Palme *et al*. 2014). This is mainly due to the misuse and abuse of human and veterinary antibiotics and their uncontrolled discharge to the aquatic environment. However, our knowledge surrounding the contribution of the discharge of these antibiotics to the environment is still lacking and are the most important gaps to fill so that strategies can be developed to prevent the further spread of these pathogens. Environments that facilitate the spread of resistant bacteria are capable of spreading bacteria that are human opportunistic pathogens (Bengtsson-Palme, Kristiansson and Larsson 2018). Effluent from WWTPs has been found to contain resistance genes that contaminate surface water (Bengtsson-Palme *et al.* 2016), which sometimes are used for small-scale agriculture, recreation, or domestic use and thus facilitate transmission from the environment to humans. For indigenous environmental bacterial strains, antibiotic exposure may contribute to the selection of resistance factors for environmental dissemination (Bengtsson-Palme, Kristiansson, and Larsson 2018). These environmental bacteria include opportunistic emerging pathogens such as *Pseudomonas* spp. (Kittinger *et al.* 2016), *S. maltophilia* (Brooke *et al.* 2017), *Aeromonas* spp. (Batra, Mathur and Misra 2016) and *Acinetobacter* spp. (Berg, Eberl and Hartmann 2005) which are investigated in this study. The potential points of pollution along the Umhlangane River (selected for this study) are illustrated in Figure 3.2 (Chapter 3) and these included a hospital, a wastewater treatment plant (WWTP), an informal housing settlement, and a veterinary clinic. The reason behind studying these tentative pollution sources was to determine if the activities along the river impact the state of the river, as the different sites (hospital, veterinary clinic) have stormwater drainage that leads directly into the river. In addition, there is the possibility of surface runoff and untreated sewage flowing into the river when the treatment plant is not functioning.

**Table 2.1 Different sources of emerging pathogens investigated in this study**

| Bacteria | Environmental sources | References |
|---|---|---|
| ***Aeromonas*** | Aquatic environments (surface water, seawater, irrigation water), raw sewage, sewage effluents, sludge, sediments. | (Janda and Abbott 2010)<br>(Olaniran, Nzimande and Mkize 2015) |
| ***Pseudomonas*** | Soil, water, oil-contaminated environments, hospital environments, and sinks and drains. | (Noura *et al*. 2009)<br>(Kittinger *et al.* 2016)<br>(Ebadi *et al.* 2017)<br>(Buhl *et al.* 2019)<br>(Lalancette *et al.* 2017) |
| ***Acinetobacter*** | Soil, water, sewage, humans, foods and animals, freshwater ecosystems; raw sewage and WWTPs, and activated sludge. | (Visca, Seifert and Towner 2011)<br>(Doughari *et al.* 2012) |
| ***Stenotrophomonas maltophilia*** | Wastewater, water column, sediments; hospitals; plant rhizospheres | Sánchez, Alonso and Martinez 2002<br>Adjidé *et al*. 2010<br>Guerci *et al.* 2019<br>Alavi *et al.* 2014 |

### 2.2.1 Untreated sewage/wastewater effluent discharge

WWTPs are reservoirs of antibiotic resistance as it is an ideal environment for antibiotic-resistant bacteria and antibiotic-resistant genes (ARG) to persist. Although the treatment process reduces the load of resistant bacteria, it has a limited impact on ARG as they are less degradable. ARG can spread between different microbial communities in the environment through horizontal gene transfer (Fouz *et al.* 2020). Selective pressure processes promote the emergence and dissemination of novel antimicrobial resistance mechanisms and new variants of antibiotic-resistant bacteria and

ARG (Zhang *et al.* 2015a). Therefore, the prevalence and survival of pathogens after treatment processes is a threat to both the receiving water bodies and ecosystems, most importantly to the receiving communities that are primarily dependent on these water sources (Luger and Brown 2010). Several environmental bacteria such as *Acinetobacter* spp., *Aeromonas* spp., and *Pseudomonas* spp. are part of microbial communities in WWTPs and are inclined to develop drug resistance (Bonomo and Szabo 2006; Zhang *et al.* 2009; Kaskhedikar and Chhabra 2010). The presence of antibiotic-resistant *Acinetobacter* species is on the increase in wastewater (Zhang *et al.* 2009). Numberger *et al.* (2019) demonstrated that the genera *Acinetobacter, Aeromonas*, and *Pseudomonas* were present in all wastewater samples. *S. maltophilia* can originate from activated sludge and sewage effluent (Adamek *et al.* 2011) and was reported to be present in municipal wastewater (Chang *et al.* 2005; Adjidé *et al.* 2010). However, in this study, most of the *S. maltophilia* isolates were found in the sediments and roots of aquatic plants (See Table 5.2.).

### 2.2.2 Informal housing settlements

Urban poverty, rapid urbanization, crowded urban settlements, and the inability of the state or the market to provide affordable housing for the urban poor, result in people residing in informal settlements across South Africa (Williams *et al.* 2018). These settlements have unique challenges because of dense human populations, poor housing infrastructure, and poor hygiene. Communities that live in these settlements lack basic waste disposal services, water, and sanitation, which leads to environmental degradation. Many settlements are situated close to rivers as this may be the only available water source (Durand 2012). Pit latrines are used as toilets and sometimes rubbish is thrown into the river (Vollmer and Grêt-Regamey 2013). This results in contamination of the environment around the settlement, and this runs off into the river. This was demonstrated in a previous study in the Western Cape, South Africa, whereby the opportunistic pathogens *Pseudomonas* (Berg River) and *Aeromonas*, *Acinetobacter*, and *Stenotrophomonas* (Plankenburg River) were found in areas densely populated by informal dwellers (Paulse *et al.* 2012). Studies investigating the pollutants in surface water around informal settlements have found various bacterial pollutants that raise health concerns for the communities around them. These include *E. coli*, *Klebsiella* species and *Enterobacter cloacae* (Leuta, Odendaal and Paulse 2020). *Acinetobacter, Aeromonas, Pseudomonas,* and *S. maltophilia* are all part of the phyla

Proteobacteria which makes up the largest percentage present in the metagenomic study by Abia *et al.* (2018), where the highest bacterial abundance and diversity was found in informal settlement samples in comparison to rural and urban settlements samples of water. This demonstrated that the microbial quality of water is adversely affected by the activities in the informal settlements. The presence of informal housing impacts the physical environment and environmental health of the communities in contact with the water source and those that may be in contact with the water further downstream.

### 2.2.3 Veterinary clinics

The veterinary clinic part of this study, which is along the river investigated, is responsible for treating sick/injured animals and rescuing domestic and farm animals, which are kept on the clinic premises. Therefore, this facility can be a source of veterinary antibiotics being released into the environment via the stormwater outlet that flows into the river or from surface runoff. Veterinary antibiotics are an integral part of animal husbandry mainly to promote the growth of livestock and for feed utilization (Teillant, Brower, and Laxminarayan 2015). The intensive use of these antibiotics has resulted in the contamination of various environmental matrices such as soil, water, and sediments (Botelho, Monteiro, and Tornisielo 2015). Once an animal is treated with antibiotics, some of the antibiotics are metabolized while unmetabolized antibiotics will be released into the environment via faeces and urine (Kumar et al. 2019). Another major issue is the development of antibiotic resistance in environmental bacterial strains due to the overuse of veterinary antibiotics (Kraemer, Ramachandran, and Perron 2019). All the bacterial species under investigation have been known to infect animals with various forms of disease (Table 2.2). Animal isolates generally show high genetic diversity and are in general distinct in their sequence types and resistance patterns compared to those found in humans. However, it cannot be excluded, that animals may occasionally play a role as a reservoir of the emerging pathogens under investigation (van der Kolk *et al.* 2019). Veterinary clinics need to implement infection control measures to prevent the release of these potential pathogens into the environment, some of which are MDR.

**Table 2.2 Animals as a source of emerging pathogens**

| Bacteria | Infections/Diseases in animals | Reference |
|---|---|---|
| ***S. maltophilia*** | Respiratory tract infection (horses)<br>UTI dogs<br>Mastitis | (Winther *et al.* 2010)<br>(Kralova-Kovarikova *et al.* 2012)<br>(Ohnishi *et al.* 2012) |
| ***Acinetobacter*** | Dogs (UTI, Throat, Skin Eczema, Abscesses)<br>Cats (Wound, urine, throat, liver)<br>Horses (nostril swabs, faeces)<br>Cows (Faecal specimens, nose swabs) | (Endimiani *et al.* 2011; Al Bayssari *et al.* 2015; Ewers *et al.* 2017; Lupo *et al.* 2017; Walther *et al.* 2018; van der Kolk *et al.* 2019) |
| ***Aeromonas*** | Faeces of Sheep, cattle, horses<br>Ready to eat fish | (Ceylan, Berktas and Ağaoğlu 2009; Abd-El-Malek 2017) |
| ***Pseudomonas*** | Otitis (Dogs)<br>Pulmonary infections (dogs, cats, bovine) | (Haenni *et al.* 2017) |

## 2.3 Multiple drug resistance

The emerging threat of MDR is a public health problem that prolongs treatment, inflicts disabilities, and increases mortality (Khan *et al.* 2020). MDR infections often have poor clinical outcomes and a high treatment cost (Thaden *et al.* 2017). This brings about the concern that the emergence of pathogens resistant to all available antibiotics, will render some infections untreatable (Lehtinen *et al.* 2019). There is an increase in the global spread of MDR Gram-negative bacteria (including all the pathogens considered in this study) that is occurring at an alarming rate (Exner *et al.* 2017). These Gram-negative bacteria enter aquatic environments from hospital wastewater that is poorly treated (Picão *et al*. 2013; Blaak *et al*. 2015) and may include *Acinetobacter, Pseudomonas, Aeromonas,* and *Stenotrophomonas maltophilia*. As these organisms are exposed to antibiotics in the water matrices, their resistance against antibiotics increases (Adegoke, Stenström, and Okoh 2017). Water environments are effective for the transfer of genes and for genetic recombination to occur. This results in the dissemination of new strains that can infect people through contact with the environment. The Centers for Disease Control and Prevention (CDC) reported on various Gram-negative MDR organisms. Of importance to this study, the CDC's findings show that of the 12 000 *Acinetobacter* infections recorded, 7 000 infections were reported to be caused by MDR strains, and this resulted in 500 deaths a year, while, of 51 000 *Pseudomonas aeruginosa* infections, 6 700 were caused by MDR strains and resulted in 440 deaths. Both fall under the category of being a serious threat (Exner et al. 2017). MDR infections from *Pseudomonas* are normally treated with carbapenems, generally considered the last line of treatment for Gram-negative bacterial infections. However, *Pseudomonas* is now becoming resistant to this class of antibiotics (Ng and Gin 2019). MDR *Acinetobacter baumannii* was detected in 53.1% of aquatic isolates and this shows the widespread contamination of natural environments (Anane *et al*. 2019). Isolates that would have been emanating from intensive care units were detected in surface water (Ng and Gin 2019). MDR *Aeromonas* was found in higher concentrations in hospital wastewater than communal wastewater (Verburg *et al*. 2019) and were resistant to eight antibiotics.

Similarly, MDR has been documented in *S. maltophilia* (Adegoke, Stenström and Okoh 2017; Li *et al*. 2019) and this has resulted in an increased mortality rate especially in individuals that are severely debilitated or immunosuppressed.

## 2.4 Virulence

Virulence is the measure of the pathogenicity of an organism (Peterson 1996), and it is directly associated with the ability to infect and cause disease (Sharma *et al.* 2017). The expression of virulence of a pathogen is affected by variables such as the number of infecting bacteria, the exposure routes into the body, host defence mechanisms, and the virulence factors of the bacteria (Peterson 1996). Opportunistic pathogens are organisms that become pathogenic after a host becomes immunocompromised (e.g., disease, wound infection, immunodeficiency, and aging) (Brown, Cornforth, and Mideo 2012). These opportunistic pathogens may emerge from environmentally indigenous organisms such as *Pseudomonas aeruginos*a (Brown, Cornforth and Mideo 2012), *Aeromonas* (Didugu *et al.* 2015), *S. maltophilia* (Denet *et al.* 2018), and *Acinetobacter* (Wong *et al.* 2017). Virulence genes (VG) /factors make these bacteria, which are ubiquitous to the natural environment, a potential health threat to humans.

To disseminate virulence, *Aeromonas* has four types of secretion systems, responsible for releasing these cell products into the extracellular environment or even directly into the host cell (Pessoa *et al.* 2019). Haemolysins, cytotoxins, enterotoxins, proteases (serine protease (*AspA*), elastase (*AhpB)]*, lipases (*Pla* and *Plc*, *Sat*), DNAses and adhesins [type IV pili, polar flagella (*FlaA* and *FlaB*)] (Agarwal, Kapoor and Kumar 1998; Cascón *et al.* 2000; Rabaan *et al.* 2001) have been identified as putative virulence factors in *Aeromonas* (Sen and Rodgers 2004). Variations of virulent gene occurrence exist in *Aeromonas* isolates between and within the genus, species, and geographical location (Ghenghesh *et al.* 2014). Several virulence factors have been identified in strains isolated from water matrices (Janda and Abbott 1998; Kingombe *et al.* 1999; Schubert 2000; Sechi *et al.* 2002).

Pathogenesis of *P. aeruginosa* is multifactorial. Virulence factors produced include secreted factors such as alkaline protease, elastase, exotoxin A, pyoverdine, pyocyanin, rhamnolipid

structural component lipopolysaccharide, pili, flagella, and biofilm formation (Murray, Rosenthal and Pfaller 2020). Production of various virulence factors is regulated by two quorum-sensing systems *las* and *rhl* (El-Mahdy and El-Kannishy 2019). An important determinant of virulence is the type III secretion system (TTSS), present in several Gram-negative bacilli. *P. aeruginosa* can produce and secrete virulence factors directly into the cytoplasm of host cells by the cell contact-mediated TTSS. The secretion of type III toxins is associated with the worst clinical outcomes in patients with ventilator-associated pneumonia and the presence of *ExoS, ExoT,* or *ExoU* secretion correlates with a greater risk of mortality (Ajayi *et al.* 2003).

Diseases caused by *A. baumannii* are due to the presence of latent VG (Darvishi 2016). Significant VG of the *A. baumannii* strains of clinical infections are colicin V production (*cvaC*), curli fibers (*csg*), siderophores like aerobactin (*iutA*), and cytotoxic necrotizing factor (*cnf*). Biofilm formation has an important role in virulence. Biofilm is a complex mixture of bacteria that is often enclosed within a thick polysaccharide layer and there are several virulence determinants associated with biofilm formation (Jahangiri *et al.* 2019). An important step in infection is the adhesion of bacterial cells to host tissues.

*S. maltophilia* exhibits its pathogenicity through various ways, which include pili/flagella/ fimbria /adhesins which contribute to adherence and colonization of biotic and abiotic surfaces. Outer membrane lipopolysaccharide plays a role in biofilm formation and resistance against antibiotics as well as complement-mediated cell killing. In addition, diffusible signal factor plays a role in QS, which in turn mediate motility, extracellular enzyme production, LPS synthesis, microcolony formation, and tolerance toward antibiotics and heavy metal ions. Finally, extracellular enzyme production such as proteases, lipases, esterase, DNase, RNase, and fibrinolysin aid pathogenicity (Looney 2005; Abbott *et al.* 2011; Brooke 2012). Although *S. maltophilia* is regarded as a low-grade pathogen it has been associated with outbreaks within hospital environments (intensive care units, respiratory units, oncology units, and surgery wards) (Brooke 2012).

## 2.5 Viability of bacteria

Bacteria are known to exist in a viable but not culturable (VBNC) state and this impairs their detection by conventional plate count methods (Li *et al.* 2014) and as a result, it can lead to the underestimation of the total viable cells in the environment (Kumar and Ghosh 2019). Pathogenic bacteria that can enter a VBNC state have a broad phylogenetic distribution, which suggests that the VBNC state may be a strategy adopted by bacteria to survive unfavourable conditions (Li *et al.* 2014). Human pathogens that exist in a VBNC state are found in water matrices, including seawater (Maalej, Denis and Dukan 2004; Dhiaf, Bakhrouf and Witzel 2008), estuarine water (Oliver 1995), stream water (Lemke and Leff 2006), lake water (Signoretto *et al.* 2004), groundwater (Cook and Bolster 2007), tap water (Pawlowski *et al.* 2011) and drinking water (Byrd, Xu and Colwell 1991).

There are various methods to determine the viability of microbial communities, but much validation is required when these methods are employed. Detection methods need to be efficient, cost-effective, practical and be able to test samples from different matrices (Kumar and Ghosh 2019). An alternate method to traditional culture-based detection is FCM combined with the staining with fluorescent dyes. This has been suggested to be a rapid method for the quantification of viable and total bacteria in water samples (Park *et al.* 2018). The LIVE/DEAD® BacLight™ Bacterial Viability Kit (BacLight kit), differentiates between live and dead cells, using membrane integrity as an indicator for cell viability (Berney *et al.* 2007; Stiefel *et al.* 2015). The BacLight kit has been well studied with researchers assessing the applicability of the kit to different bacterial strains, optimization of dye concentrations, excitation, and emission wavelengths and has shown diverse results in terms of its efficiency (Berney *et al.* 2007; Kort *et al.* 2010; Thomas *et al*. 2011; Freire *et al*. 2015; Stiefel *et al*. 2015; Park et *al.* 2018).

Many studies focus on validating pure cultures of bacteria with FCM, but limited studies are applying the technique to environmental samples such as wastewater or surface water. All bacteria under investigation in this study have been reported to exist in the VBNC state (Table 2.3) and therefore advanced methods of detection will ensure the safety of those in contact with environments in which they are present.

**Table 2.3 Species of microorganisms to enter the VBNC state**

| Species | Inducing conditions | Resuscitation conditions | Pathotype/source | Reference |
|---|---|---|---|---|
| ***Aeromonas hydrophila*** | Starvation, low temperature | Temperature shift | Human gastroenteritis septicaemia | (Maalej, Denis and Dukan 2004) |
| ***Pseudomonas aeruginosa*** | UV disinfection, low temperature, low redox potential, and oxygen limitation<br><br>Starvation, low temperature, chemicals (copper) | $TiO_2$ nanoparticles Temperature upshift.<br><br>Temperature upshifts, rich medium with copper chelator | stream water Opportunistic pathogen | (Combarros, Collado and Díaz 2016); (Lemke and Leff 2006), (Peneau, Chassaing and Carpentier 2007); (Zhang *et al.* 2015b) |
| ***Pseudomonas putida*** | NR | NR | Stream water Stream environment | (Lemke and Leff 2006) |
| ***Acinetobacter calcoaceticus*** | NR | NR | Stream water Stream environment | (Lemke and Leff 2006) |
| ***S. maltophilia*** | NR | NR | Drinking water | (Hoefel *et al.* 2005) |

NR - Not Researched

## 2.6 Methods of detection of emerging pathogens

There is no unified method to collect and analyse water for the various pathogenic organisms (Ramírez-Castillo *et al.* 2015). Some of the challenges faced with the current methods include physical differences between the pathogen groups, low pathogen concentration which requires additional enrichment and concentration steps, the presence of inhibitors in the sample, established general protocols for sample collection, and culture-independent detection methods (Ramírez-Castillo *et al.* 2015; Deshmukh *et al.* 2016). The identification and enumeration of bacteria from water samples are based on methods that are very labour intensive, time-consuming, and result in both false positive and false negative results (sometimes VBNC microorganisms cannot be recovered) (Zhao *et al.* 2017; Franco-Duarte *et al.* 2019). Alternative molecular-based methods are currently employed to identify emerging pathogens from different environmental matrices. This includes methods such as Polymerase Chain Reaction (PCR), ddPCR, real-time PCR, DNA microarray, next-generation sequencing (pyrosequencing and genomics), fluorescence *in situ* hybridization, and FCM. However, all these methods have their challenges when applied to environmental samples.

### 2.6.1 Traditional plate count on selective media

The main aim of microbiological analysis is usually to detect and enumerate a single species or a group of microorganisms in a measured sample on specific media either selective or differential. The effectiveness of this technique is limited by the cultivability of the samples and the selectivity of the medium (Amann, Ludwig, and Schleifer 1995). As found within this study, the selective media is not as discriminatory as we would expect them to be. This was validated by a comparison of the identification of isolates from traditional plate counts with PCR. Another point of consideration is VBNC bacteria, as all the organisms (*Acinetobacter*, *Aeromonas*, *Pseudomonas*, *S. maltophilia*) under investigation were reported to exist in this state (see Section 2.5). Culture-based methods have reached their limits in growing specific bacteria; however, they are still used in practice.

### 2.6.2 Matrix-Assisted Laser Desorption Ionisation -Time of Flight Mass Spectrometry

MALDI-TOF MS in combination with a reliable database is a potent method for the identification of microorganisms based on the protein fingerprints of whole cells (Benagli *et al.* 2012). MALDI-TOF MS identification of microorganisms is dependent on the detection of mass signals from biomarkers that are specific at the genus, species, or subgroup level (Benagli *et al.* 2012). There are two commercial systems (Bruker and VITEK MS) that are used for the identification of organisms (Lindgren *et al.* 2018). The VITEK MS Plus consists of two databases; the closed In Vitro Diagnosis (IVD) database, mainly used for routine identification, and the Research Use Only / Spectral Archiving and Microbial Identification System (SARAMIS) databases. A limitation of MALDI-TOF MS resides in the need for pure cultures of isolates that need to be identified (Rahi, Prakash, and Shouche 2016). Different studies in literature have reported that the identification of various bacteria is based on the available databases and their inadequacies (Espinal et al. 2012; Kishii et al. 2014; Li, Tang and Lu 2018).

*Acinetobacter* species have been identified by MALDI-TOF MS profiling however the main concern was the identification of the Acb complex (Álvarez-Buylla, Culebras, and Picazo 2012; Kishii *et al.* 2014; Wu *et al.* 2017). There have been concerns expressed regarding difficulties identifying other *Acinetobacter* species due to their close taxonomic relationship, which is highlighted in those belonging to the haemolytic clade (Nemec *et al.* 2017). These strains have identical or similar proteins which result in multiple identical mass spectrometry peaks making it difficult to discriminate. A solution to this comprises of extending databases that are available (Pailhoriès *et al.* 2015), using additional strains or type strains (Šedo *et al.* 2018) as well as the use of an alternative MALDI-TOF MS matrix or chemometric tools (Sousa *et al.* 2014). Benagli *et al*. (2012) demonstrated that MALDI-TOF MS is a useful tool for the speciation of Aeromonads, but a later study (Vávrová *et al*. 2015) found that the method is not a reliable technique for environmental isolates of *Aeromonas*. In a study, by Abdulwahab *et al*. (2015), the MALDI-TOF results were consistent with conventional phenotypic identification of isolates of *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*.

One of the main limitations in MALDI-TOF is incomplete reference databases which result in misidentifications (Li, Tang and Lu 2018). However, it must be noted that there is evidence that demonstrates the reliability of MALDI-TOF MS in comparison to traditional methods such as 16S rRNA sequencing. The method is now finding its way into environmental monitoring as a rapid tool for the detection of bacteria for monitoring and research and for identification of bacterial contaminants that impact aquatic matrices (Popović *et al*. 2017). MALDI-TOF MS is being applied more often in microbiological applications and it is beginning to replace conventional methods in laboratories (Santos *et al*. 2013).

### 2.6.3 Flow cytometry- Fluorescent *in situ* Hybridization

FCM is a single-cell analysis tool that can be used for monitoring microorganisms, and it is now a method of choice for bacterial research. It has been used in clinical (Cointe *et al.* 2017; Cunningham *et al*. 2019), and environmental studies (Nescerecka, Hammes and Juhna 2016; Tkacz, Hortala and Poole 2018). It has been reported to be a reliable technique for pathogen detection (Kennedy and Wilkinson 2017). The use of FCM combined with fluorochromes provides rapid, easy, and accurate detection and quantification of bacteria from aquatic environments, provided that there is a fluorescent probe capable of detecting specific bacteria from samples (Kennedy and Wilkinson 2017). When FCM and FISH are combined, the samples are normally fixed and labelled fluorescently before being analysed with a flow cytometer (Nettmann *et al.* 2013). FCM can be used to perform viability studies and enumerate microorganisms in environmental samples. The BacLight kit is used to enumerate bacteria (total count) and separate them into live and dead populations, (Robertson *et al.* 2019). FCM is a method of choice for bacterial research due to its advantages. The target bacteria, in this research, have been studied using FCM for various investigations, however, there are not many studies investigating the bacteria of interest in wastewater (Table 2.4).

FCM is an analytical technique and is only as good as the sample preparation and staining procedures. However, its capability to analyse many individual cells in seconds, the cell sorting function, multi-colour fluorescence detection, and sophistication of data analysis make it a tool that can improve our understanding of microbial communities in different environments.

**Table 2.4 Flow cytometric studies with target bacteria**

| Target bacteria | Studies undertaken | Reference |
|---|---|---|
| ***Aeromonas* spp.** | Viability studies in wastewater treatment | (Pianetti *et al*. 2008; Manti *et al*. 2008) |
| ***Pseudomonas* spp.** | Species-specific viability in *P. aeruginosa*, relevant to infections of the lung of CF patients. | (Rüger, Ackermann and Reichl 2014) |
| | Antimicrobial activity of engineered nanoparticle combinations (NPCs) | (Bankier *et al*. 2018) |
| ***Acinetobacter* spp.** | Flow cytometric techniques to characterise physiological states of *Acinetobacter calcoaceticus.* | (Müller *et al*. 2000) |
| ***S. maltophilia*** | The authors reported that FISH in combination with FCM is a preferable method for detection of the bacteria however no work has been done further. | (Mukherjee and Roy 2016) |

### 2.6.4 Droplet Digital PCR

ddPCR was used in this study as an advanced method of detection for the target pathogens in wastewater samples. This is a relatively new technology similar to Quantitative PCR (qPCR), it uses Taq polymerase in a PCR reaction to amplify a target DNA fragment using a primer or primer/probe assay (Hindson *et al.* 2011). This technology partitions the sample into hundreds of millions of water-in-oil droplets before thermal cycling (McDermott *et al*. 2013). These droplets are monitored for positive amplification after endpoint PCR, using fluorescent target-specific hydrolysis probes (Floren *et al.* 2015).

An advantage of ddPCR over qPCR is that it is not as sensitive to PCR inhibitors and therefore is reproducible and consistent when estimating the target DNA. Standard curves are not needed in

ddPCR (Sanders *et al*. 2011; Scollo *et al*. 2016). ddPCR can be used to detect extremely low targets from samples. This method has been used for the analysis of genetically modified organisms in animal feed and food (Morisset *et al*. 2013; Gerdes *et al*. 2016); detection and quantification of pathogens in environmental wastewater (Rothrock *et al*. 2013) and monitoring the microbial populations in soils (Kim, Jeong and Cho 2014). On surveying literature, it was noted that there are gaps in studies related to the application of this method to detect emerging waterborne pathogens such as those in this study (*Acinetobacter*, *Aeromonas*, *Pseudomonas*, and *S. maltophilia*). There are reports of applying ddPCR in environmental studies that include water. Some of these studies investigated the detection of indicator organisms such as *E. coli* (Shiga toxin-producing) in river water (Jikumaru *et al*. 2020), total coliform quantification in water environments (Ma *et al*. 2020), human faecal contamination in water quality studies (Cao, Raith and Griffith 2015) and comparative studies between ddPCR and qPCR for the detection of *Salmonella* in river sediments (Singh *et al.* 2017). Therefore, ddPCR was applied to determine the absolute quantification of the target bacteria in wastewater influent and effluent as there are no studies relating to the target bacteria and this technique. ddPCR was reported to offer an accurate and more sensitive quantification of pathogens than qPCR in environmental samples (Singh *et al.* 2017).

# Chapter 3: Methodology

## 3.1 Overview of methodology

In this chapter, an overview is given of the different methodologies used in this research. Some methodologies are common through the thesis and are described fully here, however, methods that are specific to a chapter are discussed separately in Chapter 4; Chapter 5, and Chapter 6.

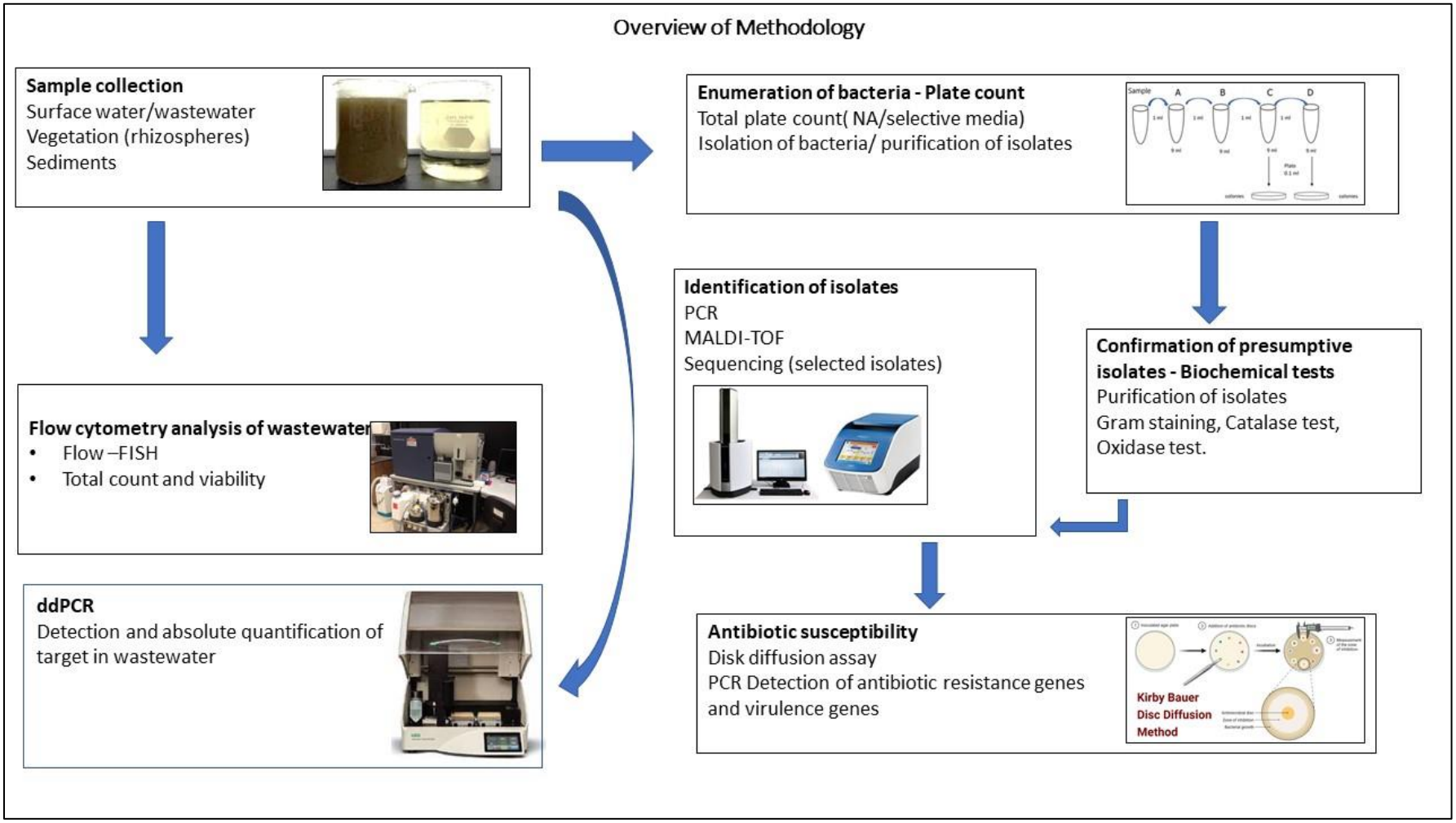


**Figure 3.1 Overview of methodologies used in the study**

## 3.2 Study area

The Umhlangane River is situated to the north of Durban, South Africa. This river flows through industrial and residential areas, and it receives effluent from a WWTP. This river flows past informal housing settlements where the water is used for daily activities, which include domestic use (washing, cooking), recreation (swimming, fishing), and small-scale agricultural activities. A hospital and veterinary clinic, which were sampling points, are located along the river (Figure 3.2).

For this study, surface water samples were collected approximately 500 m upstream and downstream from the respective four main sites (Figure 3.2; Table 3.1.) along the Umhlangane River. In addition, wastewater samples (influent and effluent) were collected from the Northern Wastewater Treatment Plant (NWWTP). The effluent from this plant is directly discharged into the Umhlangane river. The disinfection process is performed by chlorination. The treatment plant has the capacity to deal with a sewage inflow of 45 000 $m^3$ and is one of the five largest WWTPs in the city of Durban. Most of the wastewater processed is domestic (92%) while the remaining 8% is industrial wastewater (petrochemical, construction-related, cosmetic, pharmaceutical, detergent, and textile industries). The plant is currently operated using the activated sludge process configuration mode.

## 3.3 Sample collection

### 3.3.1 Surface water/wastewater sampling

Samples (1 litre) were collected from the sites listed in Figure 3.2 and Table 3.1. The WWTP – samples were collected from the influent and final effluent. Surface water samples (1 litre) were collected 500 m upstream and downstream from the following sites, Hospital (Site 1), WWTP (Site 2), Informal settlement (Site 3), and Veterinary clinic (Site 4). Grab samples of surface water ($n$=48) and wastewater ($n$=12) were taken at each sampling site, once a month, for six months from November 2016 to April 2017. Samples were collected in 1 litre sterile plastic bottles and were stored at 4°C.

### 3.3.2 Sediment and plant collection

Sediments were collected twice in October 2017 from all four surface water collection sites (Figure 3.2.; Table 3.1) following the method described by Singh *et al*. (2010). Samples were a composite of 5 sub-samples of 500 g each and were collected in sterile bags.

Plants (*n*=16) were collected at the same sampling points as the sediments. Plants were uprooted and placed in sterile plastic bags. All samples were transported on ice to the laboratory and analysed within 24 hours (h) of sampling.

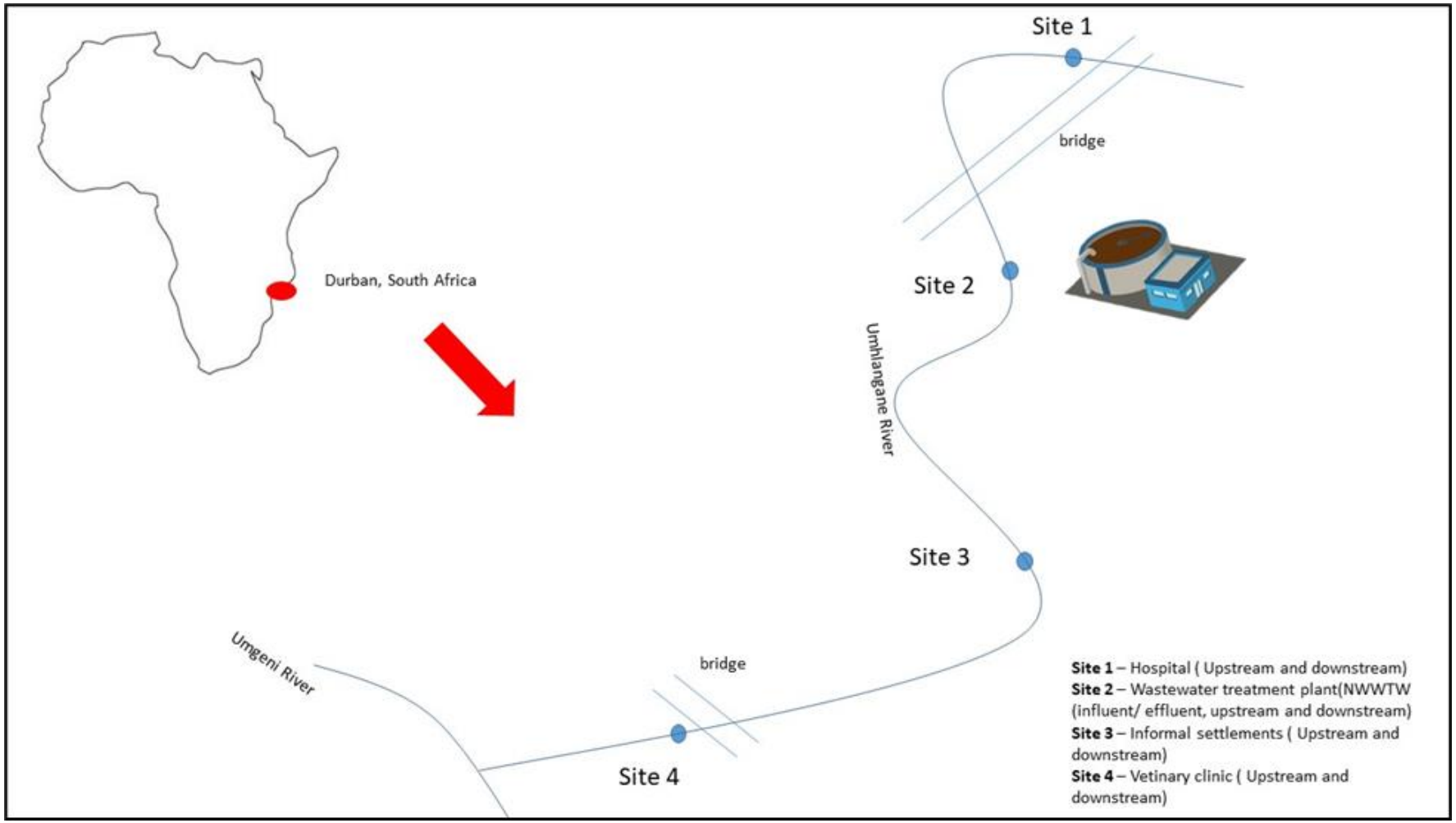


**Figure 3.2 Graphical representation of sampling sites in the study area of the Umhlangane River, Durban, South Africa**

**Table 3.1 Description of sampling sites**

| Site No | Site | Site description |
|---|---|---|
| **Site 1** | Hospital upstream | This sampling point is located upstream of a hospital and surrounded by a heavily industrialised area. |
| | Hospital downstream | Approximately, 500 meters downstream of the hospital stormwater discharge point and is surrounded by a high industry presence. |
| **Site 2** | Wastewater treatment plant upstream | Further downstream of the hospital and industrial area is the wastewater upstream sampling point. This point is 800 meters upstream of the wastewater effluent discharge point. The surrounding area is predominantly covered by residential buildings. |
| | Wastewater treatment plant downstream | This sampling point is located 500 meters downstream of the wastewater treatment effluent discharge point. |
| **Site 3** | Informal housing settlement upstream | The informal settlements are located approximately 400 meters downstream of this sampling point. |
| | Informal housing settlement downstream | This sampling point is located 500 meters downstream of the informal settlements. |
| **Site 4** | Veterinary clinic upstream | Approximately, 600 meters downstream of the informal settlements and 500 meters upstream of the stormwater discharge point of the veterinary clinic. |
| | Veterinary clinic downstream | This sampling point is located approximately, 400 meters downstream of the veterinary clinic stormwater discharge point. |

## 3.4 Isolation of opportunistic pathogens from different environmental samples

### 3.4.1 Wastewater and surface water samples

The spread plate method was used to isolate the target bacteria and to determine the total plate count from the water samples. This was based on a modified procedure (Mulamattathil *et al.* 2014). Serial dilutions ranging from $10^{-1}$ to $10^{-4}$ were prepared using sterile phosphate-buffered saline (PBS) (Sigma Aldrich, South Africa) to enable the quantification of isolates in colony-forming units (CFU) per millilitre (CFU/mL). Aliquots of 100 µL of each dilution were used to inoculate the selective medium (Table 3.3) by the spread plate method in triplicate for the isolation of *Aeromonas* spp., *Pseudomonas* spp., *Acinetobacter* spp., and *S. maltophilia*. Plate count enumeration was performed by spreading 100 µL of water sample onto Nutrient agar plates. After the incubation period (Table 3.3), presumptive counts were recorded, and the CFU/mL was determined using the following equation (APHA 2005):

$$\text{CFU/mL} = (\text{number of colonies x dilution factor})/(\text{sample volume})$$

### 3.4.2 Sediment samples

Sediments were processed using the serial dilution method. One gram of each sediment sample was added to 9 mL sterile PBS (Sigma Aldrich, South Africa) to get a 1:10 (w/v) dilution. The samples were vortexed to homogenize and bring bacteria into suspension from the sediment. This homogenized mixture was then further diluted to obtain 10-fold dilutions up to $10^{-2}$ (Singh *et al*. 2010). Aliquots (100 µL) of the dilution were plated on *Stenotrophomonas* isolation agar (Himedia, India) to isolate *Stenotrophomonas maltophilia*.

### 3.4.3 Plant rhizosphere samples

The plants that were collected were identified (Table 3.2.) by a botanist, Prof. H Baijnath (School of Life Sciences, University of Kwa-Zulu Natal) as follows: *Alternanthera sessilis* (L.) R.Br. ex DC, *Leersia hexandra* Sw. and *Ranunculus multifidus* Forssk. One gram of plant rhizosphere (cut into small pieces) was suspended in 9 mL of sterile PBS (Sigma Aldrich, South Africa) to obtain

a 1:10 dilution. This was vortexed to obtain a homogenous mixture and was further diluted. From the diluted samples, 100 µL of samples were withdrawn and then spread onto *Stenotrophomonas* selective agar to obtain isolates of *Stenotrophomonas maltophilia*.

**Table 3.2 Plants collected for the isolation of *Stenotrophomonas maltophilia***

| Plant | Ecology | Uses | Reference |
|---|---|---|---|
| ***Alternanthera sessilis* (L.) R.Br. ex DC**  | Aquatic environments | Asian cuisine, poultry feed, herbal medicine | Grard *et al*. 2006 |
| ***Leersia hexandra* Sw.**  | Shallow freshwater (marshes, swamps) | Pasture grass - cattle feed, able to accumulate heavy metals | Akobundu and Agyakwa, 1987 |
| ***Ranunculus multifidus* Forssk.**  | Freshwater environments, flood plains, coastal belts | Traditional medicines, food | Figueiredo and Smith, 2008 |

**Table 3.3 Selective media and incubation conditions used for the detection and enumeration of emerging pathogens**

| Organism | Media | Incubation condition | Presumptive colonies |
|---|---|---|---|
| ***Aeromonas*** | *Aeromonas* Isolation Agar | 24 h at 37°C | dark green, opaque with dark centres |
| ***Pseudomonas*** | Cetrimide Agar | 24 h at 35-37ºC. | Blue green/ non pigmented |
| ***Acinetobacter*** | CHROMagar *Acinetobacter* | 18-24 h at 35-37ºC. | Red |
| ***S. maltophilia*** | *Stenotrophomonas* Selective Agar | 24-48 h at 35-37°C | Cream to white colonies |
| **Total plate count (Heterotrophic bacteria)** | Nutrient Agar | 24 h at 37°C | Colour varies |

## 3.5 Enumeration of target bacteria using the spread plate method

The spread plate method was used to isolate the selected bacteria from the samples collected based on the modified procedure described in **Section 3.4**.

## 3.6 Purification of isolates

For each of the target species (*Acinetobacter*, *Aeromonas*, *Pseudomonas*, and *S. maltophilia*) representative colonies were picked from the selected culture plates based on the colonial morphology and size. The selected colonies were thereafter reinoculated onto their respective selective medium (Table 3.3.). The pure colonies were then streaked onto fresh Nutrient Agar (Oxoid, England) medium. Thereafter, single colonies were selected, inoculated into Nutrient

Broth (Oxoid, England), and were incubated for 24 h at 37$^{o}$C. The purified cultures were then used to prepare a 50% glycerol stock which was stored at -80$^{o}$C for further investigations.

## 3.7 Confirmation of presumptive isolates

### 3.7.1 Biochemical tests

Isolates were subjected to further preliminary identification tests which included Gram staining, the oxidase, and the catalase test.

#### 3.7.1.1 Gram staining

A Gram staining kit (77730, Sigma – Aldrich, South Africa) was used to stain the isolates. A drop of water was added to a slide and a colony was aseptically transferred to the slide. The culture was spread with an inoculation loop to form a thin layer on the slide. Forceps were used to hold the slide and allow it to air dry. The culture was then fixed over a Bunsen flame. The slide was then flooded with Gram's crystal violet solution for 60 seconds (s). The crystal violet solution was washed off the slide with tap water. The slide was then flooded with Gram's iodine solution, this was washed off with tap water after 60s. The next step was to decolorize the fixed culture with Gram's decolourizer solution for approximately 3s. Gram's safranin solution was used as a counterstain. After 60s, the safranin was washed off the slide and the slide was blotted with a paper towel. The slide was examined with an oil immersion objective under a microscope. All bacteria that were Gram-negative were further analysed to determine their oxidase and catalase reaction.

#### 3.7.1.2 Oxidase test

A Bactident oxidase test strip (Merck, Germany) was used for the oxidase test. The strip was allowed to make contact with the colony and a colour change to purple was indicative of a positive result.

#### 3.7.1.3 Catalase test

A pure colony of culture was transferred to a slide and a drop of 3% Hydrogen Peroxide ($H_2O_2$) was added to the fresh colony (24 h). A positive result was indicated by the production of gaseous bubbles.

## 3.8 Molecular confirmation of target bacteria

PCR was used to amplify and detect the presence of specific gene sequences in each of the presumptive target species (*Acinetobacter, Pseudomonas, Aeromonas,* and *S. maltophilia*).

### 3.8.1 DNA extraction

DNA extraction was carried out using the boiling method as described by Jyoti *et al*. (2011). The isolates which were stored as glycerol stocks were streaked onto Nutrient Agar. After 24 h, single colonies of the culture were transferred into a 2 mL microcentrifuge tube with 500 µL of sterile distilled water. This was boiled in a heating block at 95°C for 15 minutes (min) and then centrifuged at 14,000 rpm for 5 min at 4°C. The upper layer of supernatant from the tube was removed and to this, 25 µL of 0.3M sodium acetate (pH = 5.2) and ice-cold ethanol was added to allow for the precipitation of DNA. The precipitated DNA was pelleted by centrifugation at 14 000 rpm for 15 min. The DNA pellet was washed twice with 70% ethanol and resuspended in 250 µL of TE buffer. The template DNA was stored at -20°C and used for all PCR reactions.

### 3.8.2 PCR identification of target organisms

Genus-specific PCR was performed to confirm the identification of presumptive isolates. Primers specific for each of the target genus (*Acinetobacter*, *Pseudomonas, Aeromonas*) and *S. maltophilia* were selected (Table 4.1. and Table 5.1.). The PCR reactions were performed using a Biorad T100 thermocycler (Biorad, USA). The PCR mixture was of a total volume of 25 µL. This comprised of 12.5 µL DreamTaq Hotstart master mix (Thermofisher, South Africa), 1 µL forward primer, 1 µL reverse primer, 5.5 µL nuclease-free water, and 5 µL template DNA at a concentration of 5 nanograms (ng).

### 3.8.3 Electrophoresis of PCR products

The amplified PCR products were visualised with agarose gel (2%) electrophoresis which was stained with GelRed® (Biotium, USA). A 100-base pair (bp) / 50 bp DNA Ladder (Fermentas, USA) was used to measure the size of the amplicons. Electrophoresis was conducted for 90 min at 65V (Power Pac Basic, Bio-Rad) using 1x TAE (Tris Acetate Ethylene diamine tetra-acetic acid) buffer (40mM Tris, 1mM EDTA, and 20mM glacial acetic acid, pH 8.0). The gel image was viewed using the GelDoc Imaging System (BioRad, South Africa).

## 3.9 Identification of isolates using MALDI-TOF MS.

This is described in detail in **Chapter 4: Section 4.2.5.** The workflow for the identification of bacteria using the MALDI-TOF MS is shown in Appendix B.

## 3.10 Antibiotic resistance profiling

The methodology is detailed in **Chapter 4: Section 4.2.6 (**Antibiotic resistance profiling**); Chapter 4: Section 4.2.7** Multiple antibiotic resistance (MAR) index and **Chapter 4: Section 4.2.3. (**Virulence and antibiotic-resistant marker). **A detailed workflow for the antibiotic resistance profiling is provided in Appendix C.**

## 3.11 Flow cytometry methodology

The FCM methodology is described in detail in **Chapter 6: Section 6.2.5.** The figure below diagrammatically represents the process followed for the analysis of wastewater using Flow Cytometry combined with Fluorescent-*In situ* hybridization (Flow-FISH) Flow-FISH.

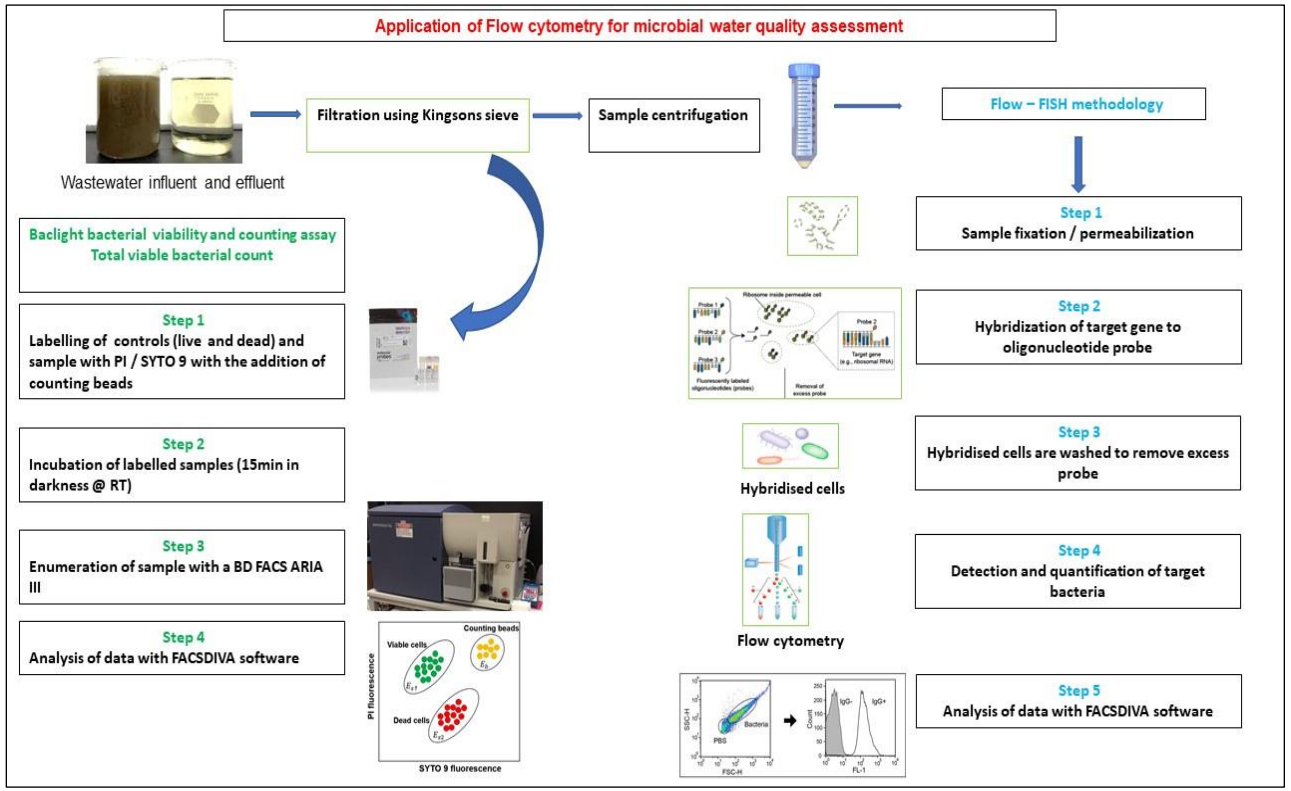


**Figure 3.3 Workflow of BacLight bacterial viability and counting assay and the Flow-FISH assay**

## 3.12 Analysis of wastewater samples using droplet digital PCR

This method is described completely in **Chapter 6: Section 6.2.4.** Each step of the assay is represented in Figure 3.4.

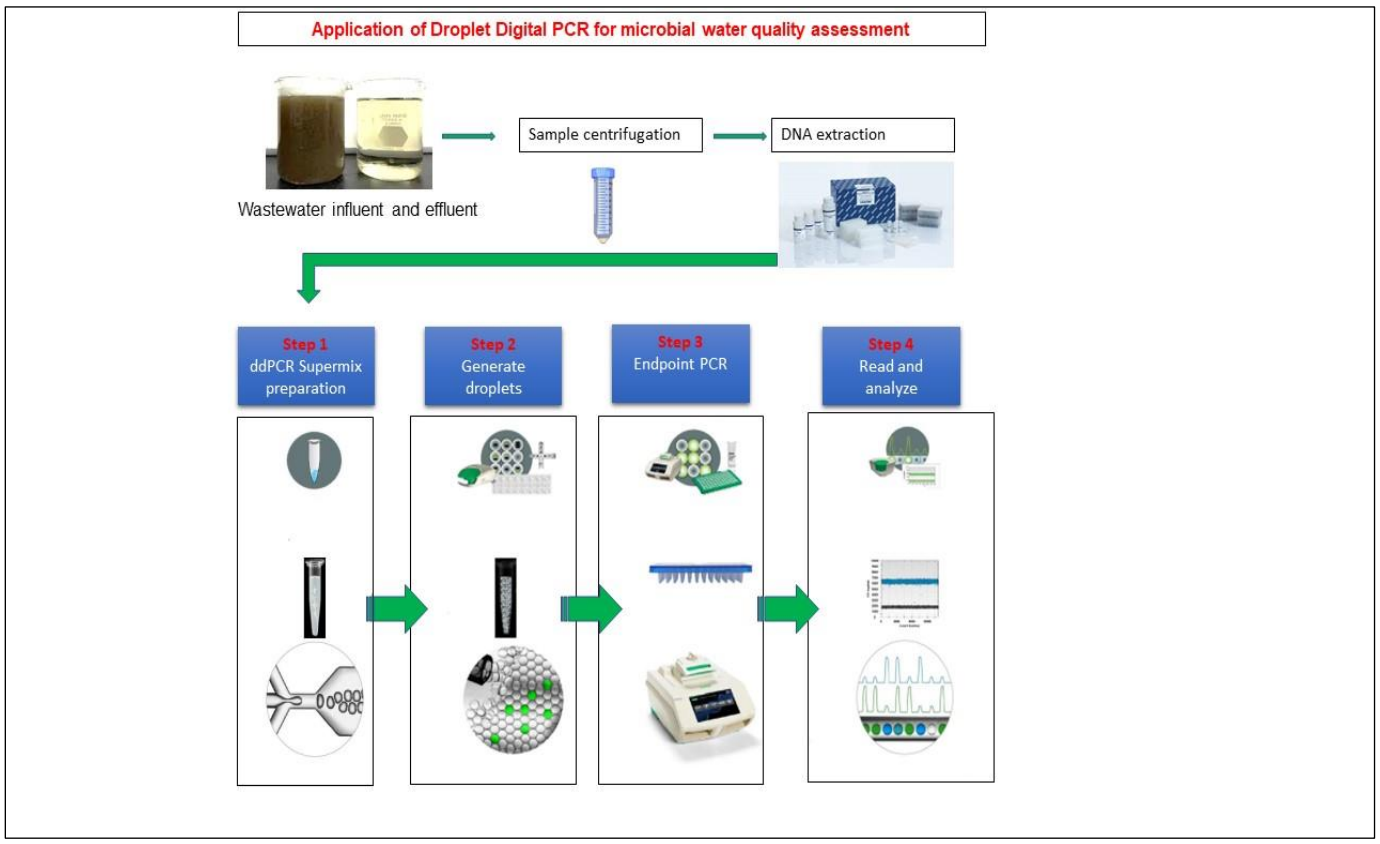


**Figure 3.4 The schematic on the workflow for the analysis of wastewater analysis using ddPCR**

# CHAPTER 4: Molecular and MALDI-TOF MS identification, Antibiotic Resistance and Virulence Gene Profiling in *Aeromonas* and *Pseudomonas* Species

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

## 4.1 Introduction

Waterborne bacterial pathogens are a major public health concern worldwide, not only due to the morbidity and mortality they cause but also due to the high cost of treatment and prevention (Ramírez-Castillo *et al*. 2015). In developing countries, poverty-stricken communities and residents rely on rivers, streams, and ponds for their daily water needs (Mackintosh and Colvin 2003; Braune and Xu 2008; Nkuna, Mamakoa and Mothetha 2014). These natural sources of water are often contaminated by waterborne opportunistic pathogenic microorganisms (Mema 2010), including species of *Pseudomonas* (Igbinosa *et al*. 2012a; Mulamattathil *et al*. 2014) and *Aeromonas* (Olaniran, Nzimande and Mkize 2015). These bacteria may have profound health implications for immunocompromised individuals and are, therefore, a serious challenge in a country like South Africa with over 12% of the population in this category (Statistics South Africa, 2017).

*Aeromonas* spp. has been reported as waterborne opportunistic pathogens which are widely distributed in soil, food, and aquatic environments (Janda and Abbott 2010). *Aeromonas* spp. have been isolated from wastewater (Martone-Rocha *et al*. 2010), rivers, aquaculture environments (Evangelista-Barreto *et al*. 2010), and potable water systems (Figueira *et al.* 2011). This species is mainly associated with gastrointestinal diseases affecting the elderly, children, and immunocompromised people (Daskalov, 2006). Common *Aeromonas* spp. infections are wound infections, meningitis, septicaemia, pneumonia, and endocarditis (Bhatia *et al.* 2017). *Aeromonas*

spp. associated with human pathogenicity are *A. hydrophila, A. caviae,* and *A. veronii biovar sobria* (Janda and Abbott 2010). Other species such as *A. jandei, A. schubertii* and *A. veronii biovar veronii* are sometimes responsible for culture-based confirmed infections of sepsis (Janda and Abbott 1998). *Aeromonas* spp. display absolute resistance against ampicillin and oxacillin, which illustrate the intrinsic resistance of *Aeromonas* to *β*-lactam antibiotics related to *β*-lactamase genes (Igbinosa and Okoh 2013). Some isolates of *Aeromonas* spp. display resistance against the antibiotic tetracycline (Igbinosa *et al.* 2012a). Virulence in *Aeromonas* species is multifactorial, several putative VG has been identified in this bacterium encoding for haemolysins (*hyl*), enterotoxins (*ast, act*), proteases (*AspA*), lipases (*lip*), and aerolysins (*aer*) (Sen and Rodgers, 2004; Olaniran, Nzimande and Mkize 2015).

Along with *Aeromonas* spp., pathogenic *Pseudomonas* spp., which are found naturally in soil, aquatic environments, and various other natural habitats, are a serious health risk to exposed individuals (Ruiz *et al.* 2004). *Pseudomonas* spp. can survive in low and high nutrient environments (Mena and Gerba 2009) and has been reported to harbour multiple resistance genes (Juan and Oliver 2010; Pfeifer, Cullik and Witter 2010; Fariñas and Martínez-Martínez 2013). The most common species of the genus *Pseudomonas* is *P. aeruginosa* which is known to cause various diseases such as pneumonia (Garau and Gomez 2003), urinary tract infections (Bitsori *et al.* 2012), bacteraemia (Tam *et al.* 2010), and chronic lung infections in patients with cystic fibrosis (Bjarnsholt *et al.* 2009; Mena and Gerba 2009). *P. fluorescens* has been found in many clinical samples linked to infections in the bloodstream (Scales *et al.* 2014). *P. putida* has emerged as a multi-drug and carbapenem-resistant strain (Kim *et al.* 2012), causing bacteraemia in soft tissue injuries (Yoshino *et al.* 2011; Thomas *et al.* 2013). As a pathogen, *Pseudomonas* spp. are difficult to eliminate from the environment due to their resistance against various antibiotic classes including aminoglycosides, fluoroquinolones, and *β*-lactams (Livermore 2002).

Many reports describe the characterisation of virulence and antibiotic resistance in *Pseudomonas* spp. and most focus only on *P. aeruginosa* (Bradbury *et al*. 2010; Fazeli and Momtaz, 2014; Faraji *et al*. 2016). Other studies have shown the presence of VG such as *toxA* (Michalska and Wolf, 2015), *exoS* (Yousefi-Avarvand *et al.* 2015), *ecfX* (Lavenir *et al.* 2007), and *lasI* (Bratu, Gupta and Quale 2006) in *Pseudomonas* spp. which are a concern for public health. It is highly unlikely to

solve the problems associated with antibiotic resistance in pathogens or infection control depending only on the studies conducted in clinical settings; hence the interlinkage to the environment must be considered, especially in water matrices (Adegoke *et al.* 2016).

Anthropogenic activities are known sources of surface water pollution resulting in the decrease of water quality (Adeosun, Adams and Amrevuawho 2016). Given the growing interest in understanding the possible role played by several factors such as human activities, industrial discharges, hospital effluents, and WWTPs. in the emergence of antibiotic-resistant bacteria, this study focused on the isolation, quantification, antibiotic resistance, and virulence profiling of *Aeromonas* spp. and *Pseudomonas* spp. from wastewater and surface water in Durban, South Africa.

## 4.2 Methodology

### 4.2.1 Study area and sampling points

The study area included NWWTP (global positioning system coordinates: -29.7955417, 30.9978762) and the Umhlangane river located in Durban, South Africa. This has been described in **Chapter 3: Section 3.2**. of this thesis. One-litre water samples (influent and effluent) were collected from influent and effluent points of NWWTP. Surface water samples were collected approximately 500 m upstream and downstream from the eight sampling points (Figure 3.2, Table 3.1.). A full description of the sample collection is listed in **Chapter 3: Section 3.3.1**.

### 4.2.2 Isolation and characterisation of the bacterial isolates

Samples were analysed using the spread plate technique as described in **Chapter 3: Section 3.4.1.** The selective medium with incubation conditions and the selection of presumptive colonies are listed in Table 3.3. The colonies were enumerated as described in **Chapter 3: Section 3.4.1.** and recorded as CFU/mL of sample. The presumptive isolates with a typical appearance on the respective medium (dark green for *Aeromonas* and cream-colored colonies for *Pseudomonas*) were further streaked onto fresh selective media as described in **Chapter 3: Section 3.6**. The isolates were then characterised by Gram staining (**Chapter 3: Section 3.7.1.1**.). Gram-negative cultures

were further analysed for oxidase and catalase production (**Chapter 3: Section 3.7.1.2.** and **3.7.1.3** respectively).

### 4.2.3 Identification of the isolates and detection of antibiotic-resistant and virulence markers using PCR

After initial screening and characterisation, the isolates were identified to genus level using 16S rRNA identification facilitated by PCR amplification (**Chapter 3: Section 3.8.2).** Genomic DNA (gDNA) was extracted from individual isolates (**Chapter 3: Section 3.8.1**) and was used as a template and 16S rRNA genus-specific primers were used to amplify the *gyrase B* gene (*gyrB*) for *Aeromonas* (Küpfer *et al.* 2006) and the *PSE* gene for *Pseudomonas (*Robertson *et al.* 2014). The PCR cycling conditions used were 10 min at 95°C, 20 s at 95°C, 30 s at 56°C, and 30 s at 72°C (45 cycles) and a final extension of 10 min at 72°C for *Aeromonas* and 10 min at 95°C followed by 40 cycles of 95°C for 30 s and 1 min at 60°C for *Pseudomonas*. A 25 µL PCR reaction mixture contained 50 ng template DNA, 0.4 µM of each primer set, 1x Maxima Hotstart PCR master mix (Thermo Fisher Scientific, Walthan, MA, USA), and nuclease-free water. *Aeromonas hydrophila,* American Type Culture Collection (ATCC) 7966, and *Pseudomonas aeruginosa* ATCC 27853 strains were used as positive controls. The electrophoresis of amplified products is described in Chapter 3: Section 3.8.3. To detect the ARG and VG in individual isolates, gDNA was extracted and PCR was performed using the conditions described above. The PCR conditions used were identical for all the genes except for the annealing temperatures. The gene names, primer sequences, and annealing temperature used are shown in Table 4.1.

**Table 4.1 Primers used in the study, gene amplicon size and annealing temperature for PCR**

| Gene | Sequence (5'-3') | Amplicon size | Annealing Temp (°C) | Reference |
|---|---|---|---|---|
| ***PSE*** | F-ACTTTAAGTTGGGAGGAAGGG<br>R-5ACACAGGAAATTCCACCACCC | 251 | 60°C | (Bergmark *et al.* 2012) |
| ***Gyrb*** | F-GAAGGCCAAGTCGGCCGCCAG<br>R-ATCTTGGCATCGCCCGGGTTTTC | 198 | 56°C | (Robertson *et al.* 2014) |
| ***bla$_{TEM}$*** | F-AGGAAGAGTATGATTCAACA<br>R-CTCGTCGTTTGGTATGGC | 535 | 55°C | (Wang *et al.* 2006) |
| ***bla$_{OXA}$*** | F-TGAGCACCATAAGGCAACCA<br>R-TTGGGCTAAATGGAAGCGTTT | 311 | 53°C | (Kuo *et al.* 2010) |
| ***bla$_{AmpC}$*** | F-GGTATGGCTGTGGGTGTTA<br>R-TCCGAAACGGTTAGTTGAG | 882 | 53°C | (Yang *et al.* 2008) |
| ***tet$_C$*** | F-GGTTGAAGGCTCTCAAGGGC<br>R-GGTTGAAGGCTCTCAAGGGC | 505 | 65°C | (Agersø and Sandvang 2005) |
| ***mcr-1*** | F-GGGCCTGCGTATTTTAAGCG<br>R-CATAGGCATTGCTGTGCGTC | 183 | 55°C | (Hembach *et al.* 2017) |
| ***Sul1*** | F-CGGCGTGGGCTACCTGAACG<br>R-GCCGATCGCGTGAAGTTCCG | 433 | 60°C | (Kerrn *et al.* 2002) |
| ***Sul2*** | F-GCGCTCAAGGCAGATGGCATT<br>R-GCGTTTGATACCGGCACCCGT | 293 | 60°C | Kerrn *et al.* 2002) |
| ***Sul3*** | F-TCAAAGCAAAATGATATGAGC<br>R-TTTCAAGGCATCTGATAAAGAC | 787 | 55°C | (Heuer and Smalla 2007) |
| **16S-27F**<br>**16S-1492R** | F-AGAGTTTGATCMTGGCTCAG<br>R-CGGTTACCTTGTTACGACTT | 1300 | 55°C | (Marchesi *et al.* 1998) |

| | | | | |
|---|---|---|---|---|
| ***ToxA*** | F-GACAACGCCCTCAGCATCACCAGC<br>R-CGCTGGCCCATTCGCTCCAGCGCT | 367 | 66°C | (Mulamattathil *et al*. 2014) |
| ***ExoS*** | F-GCGAGGTCAGCAGAGTATCG<br>R-TTCGGCGTCACTGTGGATGC | 118 | 58°C | (Ajayi *et al*. 2003) |
| ***EcfX*** | F-ATGGATGAGCGCTTCCGTG<br>R-TCATCCTTCGCCTCCCTG | 528 | 58°C | (Lavenir *et al*. 2007) |
| ***LasI*** | F-ATGATCGTACAAATTGGTCGGC<br>R-GTCATGAAACCGCCAGTCG | 605 | 56°C | Bratu, Gupta and Quale 2006) |
| ***Aer*** | F-CCTATGGCCTGAGCGAGAAG<br>R-CCAGTTCCAGTCCCACCACT | 431 | 64°C | (Igbinosa *et al*. 2013) |
| ***Alt*** | F-CCATCCCCAGCCTTTACGCCAT<br>R-TTTCACCGAGGTGACGCCGT | 338 | 63°C | (Martínez *et al*. 2009) |
| ***Ast*** | F-ATGCACGCACGTACCGCCAT<br>R-ATCCGGTCGTCGCTCTTGGT | 260 | 55°C | (Martínez *et al*. 2009) |
| ***Fla*** | F-TCCAACCGTYTGACCTC<br>R-GMYTGGTTGCGRATGGT | 608 | 55°C | (Sen and Rodgers 2004) |

### 4.2.4 Sequencing and analysis

The PCR products were excised from the agarose gel and purified using the Zymoclean Gel DNA Recovery Kit (#D4001, Zymo Research, South Africa) and this was sent for sequencing to Inqaba Biotech, Pretoria, South Africa. The sequences obtained were then searched for homology at National Centre for Biotechnology Information nucleotide Basic Local Alignment Search Tool (Altschul *et al.* 1990).

### 4.2.5 Identification of isolates using MALDI-TOF MS

For the identification of the isolates using MALDI-TOF MS, the samples were shipped on ice to the Centre for Antibiotic Resistance Research (CARe) at the University of Gothenburg, Sweden.

The PCR positive isolates were cultured overnight on Blood agar. These were transferred onto a VITEK MS-DS slide (BioMérieux, France) using a 1 $\mu$L loop. Immediately after spotting, 1 $\mu$L of matrix α-cyano-4-hydroxycinnamic acid matrix solution (BioMérieux, France) was added. The slides were dried at room temperature and loaded into a VITEK mass spectrometry system (BioMérieux, France). The mass spectra were analysed using the VITEK MS databases IVD *v.*3.0 and SARAMIS *v.*4.15.

### 4.2.6 Antibiotic resistance profiling of *Aeromonas* spp. and *Pseudomonas* spp. isolates

Antibiotic susceptibility testing was performed using the standard Kirby-Bauer disk diffusion method (Tao *et al.* 2010). Positively identified isolates (*Aeromonas* spp. and *Pseudomonas* spp.) were cultured, standardized to a 0.5 McFarland standard, and swabbed on Mueller Hinton agar plates. A panel of 12 antibiotics of clinical relevance was tested against isolates by applying antibiotic discs (Mast Diagnostics, United Kingdom) to the surface of Mueller Hinton agar plates according to the manufacturer's instructions. Seven classes of antibiotics were analysed, these were $\beta$-lactams (ampicillin 20 $\mu$g, ceftazidime 30 $\mu$g, and cefixime 5 $\mu$g), polymyxins (polymyxin B 300 units and colistin 25 $\mu$g), (fluor) quinolones (ciprofloxacin 5 $\mu$g, levofloxacin 5 $\mu$g and ofloxacin 5 $\mu$g), tetracyclines (minocycline 30 $\mu$g), carbapenems (meropenem 5 $\mu$g and imipenem 10 $\mu$g) and sulphonamides (trimethoprim-sulphamethoxazole 25 $\mu$g). The diameter of the zone of inhibition was measured to the nearest millimetre and this was used to determine the level of susceptibility or resistance. The results were interpreted according to the standards of the Clinical Laboratory and Standards Institute (CLSI) (CLSI, 2016; CLSI, 2017).

### 4.2.7 Multiple antibiotic resistance index

The isolates showing resistance against three or more antibiotics were classified as MDR and the Multi Antibiotic Resistance (MAR) index was calculated, according to the equation below (Blasco, Esteve and Alcaide 2008).

$$\mathrm{MAR} = \mathrm{a}/\mathrm{b},$$

Where ‘a’ is the number of antibiotics to which the isolate was resistant, and ‘b’ is the total number of antibiotics against which individual isolate was tested. The MAR index is a widely used tool to evaluate antibiotic resistance and its associated health risks (Riaz, Faisal and Hasnain 2011). An isolate with a MAR index greater than 0.2 is considered to be from a high-risk area, with possible exposure to high doses of antibiotics over a period (Paul *et al*. 1997; Odjadjare *et al*. 2012). While a MAR index less than or equal to 0.2, is considered as an intrinsic strain resistance against the antibiotic without previous exposure to such antibiotics (Saka, Adeyemo and Odeseye 2017).

### 4.2.8 Statistical analysis

Data analysis for plate counts was performed using GraphPad Prism *v*.5.0. The two-tailed test and two-way ANOVA were used to determine the difference in means between antibiotic-resistant strains to antibiotics as well as differences between the genera for individual antibiotics at a 95% confidence interval. Differences in frequencies of resistance phenotype (RP), VG, and ARG among groups were evaluated using chi-square tests ($\chi^2$) on contingency tables with a significance level of $p = 0.05$. Analyses were performed in IBM SPSS Statistics *v*.25.

## 4.3 Results and discussion

### 4.3.1. Isolation and enumeration of *Aeromonas* spp. and *Pseudomonas* spp. from wastewater and surface water samples

*Aeromonas* spp. and *Pseudomonas* spp. were present in all samples analysed in this study with varying concentrations. The average counts ($log_{10}$ CFU/mL) for presumptive *Aeromonas* spp. (6.2 ±0.25 $log_{10}$ CFU/mL) and *Pseudomonas* spp. (3.19±1.46 $log_{10}$ CFU/mL) in the influent wastewater, samples were higher than the effluent samples (*Aeromonas* spp.: 2.91±1.12 $log_{10}$ CFU/mL; *Pseudomonas* spp.: 1.72±1.40 $log_{10}$ CFU/mL), which demonstrated greater than 94% removal efficiency (Table 4.2.). This difference in concentration of both *Pseudomonas* spp. and *Aeromonas* spp. in influent and effluent samples were statistically significant. This shows that the treatment process contributed significantly to the reduction in the concentration of both *Aeromonas* spp. and *Pseudomonas* spp.

Different removal efficiencies for wastewater treatment ranging from 95% to 98% for faecal indicator organisms have been reported previously (Barrios-Hernández *et al*. 2020). Specifically, Numberger *et al.* (2019) reported prevalence of *Pseudomonas* spp. and *Aeromonas* spp. of 5.8% and 1.1%, respectively in untreated wastewater. After mechanical treatment, followed by a biological phosphate elimination process, there was a reduction in the prevalence of *Pseudomonas* spp. and *Aeromonas* spp. in the effluent (1.1% and 0.02% respectively). The removal efficiency in this study therefore can be compared to their findings as in both studies the overall abundance was greatly reduced but not completely removed.

There was no statistical difference in both *Pseudomonas* spp. and *Aeromonas* spp. concentrations upstream and downstream of the wastewater effluent discharge point (Table 4.2.). However, the contribution of other sites, such as the hospital (site 1) and the veterinary clinic (site 4) was found to impact the concentration of these bacteria in the surface water (river samples). For instance, the impact of the veterinary clinic's (site 4) contribution to the increased loads in surface water was evident due to an increase of 0.6 $log_{10}$ CFU/mL observed in downstream samples (*Aeromonas* spp.*:* 3.1±0.3 $log_{10}$ CFU/mL, *Pseudomonas* spp.*:* 1.7±1.4 $log_{10}$ CFU/mL) than the upstream

samples (*Aeromonas*: 2.5±0.8, *Pseudomonas*: 1.1±1.3). This could be due to a storm-water outlet that leads to the river just before the downstream sampling point. Similarly, at the hospital sampling point (site 1), higher concentrations of *Aeromonas* spp. (3.1±0.7 $\log_{10}$ CFU/mL) and *Pseudomonas* spp. (1.2±1.4 $\log_{10}$ CFU/mL) were found in the downstream samples (Table 4.3.). The hospital storm-water drainage empties into the river approximately 50 meters upstream from the hospital downstream sampling point, which could have contributed to this notable increase in bacterial concentrations. The impact of hospital wastewater or stormwater on surface water contamination with these bacteria (*Aeromonas* spp. and *Pseudomonas* spp.) has been reported previously (Mena and Gerba 2009; Fisher *et al*. 2015, Batrich *et al*. 2019). For instance, Mena and Gerba, 2009 reported that urban runoff was thought to be a source of *Pseudomonas* spp., while it was reported by Warburton, Bowen, and Konkle (1994) to be an indicator of surface run-off contamination. Olds *et al*. (2018) has reported that stormwater systems are sometimes contaminated by sewage due to leaking sewage infrastructure infiltrating the stormwater or illicit connections leading to the discharge of untreated sewage into the surface water. It was further noted that the informal settlement (Figure 3.2. – Site 3) did not show any adverse impact on the concentration of these bacteria in the receiving river. Concentrations of the bacteria upstream of the informal settlement were higher (*Pseudomonas* spp.: 1.1±1.7 $\log_{10}$ CFU/mL; *Aeromonas* spp.: 3.3±0.4 $\log_{10}$ CFU/mL) than downstream (Table 4.3.).

**Table 4.2 Mean Log reduction/removal efficiency of the treatment plant (NWWTP) over a six-month sampling period (Nov 2016 – April 2017)**

| **Bacteria** | **$\log_{10}$ (CFU/mL)** | | **Reduction efficiency (%)** | ***p* values** |
|---|---|---|---|---|
| | **Influent** | **Effluent** | | |
| ***Aeromonas* spp.** | 6.27±0.25 | 2.91±1.12 | 99.8 | $\leq 0.05$ |
| ***Pseudomonas* spp.** | 3.19±1.46 | 1.72±1.40 | 94.7 | $\geq 0.05$ |

**Table 4.3 $Log_{10}$ CFU/mL of *Aeromonas* spp. and *Pseudomonas* spp. in surface water sampling sites (average over six months)**

| Sampling Site | $Log_{10}$ CFU/mL | | | |
|---|---|---|---|---|
| | *Aeromonas* spp.* | | *Pseudomonas* spp.* | |
| | Upstream | Downstream | Upstream | Downstream |
| **Hospital (Site 1)** | 3.0±0.9 | 3.1±0.7 | 0.6±7.7 | 1.2±1.4 |
| **WWTP (U/S & D/S of site 2)** | 3.3±1.0 | 2.7±1.0 | 1.7±1.9 | 1.8±1.0 |
| **Informal Housing (Site 3)** | 3.3±0.4 | 2.8±1.4 | 1.1±1.7 | 0.6±1.1 |
| **Veterinary clinic (Site 4)** | 2.5±0.8 | 3.1±0.3 | 1.1±1.3 | 1.7±1.4 |

U/S – Upstream; D/S – Downstream; **p*-value > 0.05

### 4.3.2 Confirmation of *Aeromonas* spp. and *Pseudomonas* spp. isolates

The isolates were further confirmed by PCR and by using MALDI-TOF MS. Approximately, 81% of the presumptive *Pseudomonas* isolates (*n*=55) confirmed by PCR were further identified by MALDI-TOF MS to the species level (Table 4.4.). These comprised of *P. putida* (51%), *P. aeruginosa* (26%), *P. mendocina* (16%), *P. alcaligenes* (5%) and *P. oleovorans* (2%). A total of 12% of the isolates were identified as belonging to other genera (*Providencia, Ochrobactrum, Enterobacter*, *Streptococcus*).

In this study, the identification of the target bacteria by MALDI-TOF MS was comparable to 16S rRNA sequencing. However, there were discrepancies in identification in comparison to the above-mentioned methods to PCR. In certain instances, PCR identifications of bacterial isolates have been reported to be false positive. This could be attributed to the sensitivity or specificity of the primer and sometimes could be because of contamination (Ruiz-Villalba *et al*. 2017).

The MALDI-TOF MS analysis of the PCR positive *Aeromonas* spp. (*n*=95) showed that only 65% of isolates belonged to the genus, *Aeromonas* (Table 4.4.). The remaining 24% belonged to other genera (*Pseudomonas, Enterobacter, Citrobacter,* and *Acinetobacter*) and 11% were found non-

viable on receipt for analysis and could not be analysed by MALDI-TOF MS. Previously, misidentification of isolates through MALDI-TOF MS was reported due to analysis of nonaxenic cultures (Anderson *et al.* 2012) and technical errors during sample preparation (Cherkaoui *et al.* 2011). The speciation of the isolates belonging to the *Aeromonas* genus (*n*=62) was found to be *A. hydrophila*/*caviae* (58%), *A. caviae* (13%), *A. hydrophila*/*caviae*/*sobria* (11%), *Aeromonas* spp. (8%) and *A. veronii/sobria* (10%). A reference strain *A. hydrophila,* ATCC 7966 was analysed and identified by MALDI-TOF MS as *A. hydrophila/caviae.* This shows that MALDI-TOF MS could not differentiate between these two species. This inconclusive identification was common in other isolates, such as *A. hydrophila*/*caviae*/*sobria* and *A. veronii/sobria.* Deng *et al.* (2014) reported that there was unclear species identification within the *Aeromonas* genus using MALDI-TOF MS and suggested that updating the existing databases may assist in better discrimination between the species. Timperio *et al*. (2017) further concluded that MALDI-TOF MS can be chosen for identification owing to its easy and rapid sample preparation for accurate identification up to species level, however, the database needs to be updated with a larger number of environmental strains. Therefore, a concise database is required for the accurate identification of closely related organisms that have similar spectrums when using the MALDI-TOF MS platform for bacterial identification (Croxatto, Prodhom and Greub 2012).

**Table 4.4 Identification of the isolates from the study**

| Method | *Aeromonas* spp. | | *Pseudomonas* spp. | |
|---|---|---|---|---|
| **PCR** | *n*=95 | | *n*=68 | |
| **Identified other than genus (%)** | 24 | | 12 | |
| **MALDI-TOF MS** | *n*=62 | % | *n*=55 | % |
| | *A. hydrophila* / *caviae* | 58 | *P. putida* | 51 |
| | *A. caviae* | 13 | *P. aeruginosa* | 26 |
| | *A. hydrophila* / *caviae* / *sobria* | 11 | *P. mendocina* | 16 |
| | *A. veronii*/*sobria* | 10 | *P. alcaligenes* | 5 |
| | *Aeromonas* spp. | 8 | *P. oleovorans* | 2 |

### 4.3.3 Antimicrobial resistance profile of *Aeromonas* spp. and *Pseudomonas* spp. isolates

The antimicrobial resistance analysis (Table 4.5.) shows that the *Aeromonas* isolates were resistant to trimethoprim-sulphamethoxazole (100%), ampicillin (76%), polymyxin B (56%), and colistin (42%). Lower levels of resistance (8%) were displayed to levofloxacin, meropenem and imipenem; 5% to third-generation cephalosporin's cefixime and ceftazidime; 3% to minocycline and 2% to ciprofloxacin, while none of the isolates was resistant to ofloxacin. Similar resistant profiles have been reported in previous studies for *Aeromonas* spp. (Goñi-Urriza *et al*. 2000b; Pérez-Valdespino, Fernández-Rendón and Curiel-Quesada 2009, Odeyemi and Ahmad, 2017). Absolute resistance of *Aeromonas* spp. to ampicillin and oxacillin was reported in a previous study in Durban, South Africa (Olaniran, Nzimande and Mkize 2015), and the resistance was attributed to lactamase activity in resistant isolates (Igbinosa *et al.* 2012a). The resistance of the *Aeromonas* isolates

observed in this study could be attributed to the intrinsic resistance of these isolates due to *β*-lactamase production (Figueira *et al*. 2011). *Aeromonas* isolates from this study displayed resistance against polymyxin B, as well as resistance against some quinolones (levofloxacin, and ciprofloxacin), third-generation cephalosporins (ceftazidime, cefixime), and meropenem. The results from this study are in contrast to a study conducted in the United States where *Aeromonas* spp. showed susceptibility to third-generation cephalosporins (>90%) and almost all isolates were susceptible to quinolones (Zhiyong, Xiaojou and Yanyu 2002).

*Pseudomonas* spp. isolates from this study displayed resistance (21% and 7%) to the carbapenems (meropenem and imipenem). Carbapenem-resistant *P. aeruginosa* isolates have been included in a list of antibiotic-resistant priority pathogens that pose a threat to human health (WHO, 2017). This implies that there is an urgent need for new antibiotics for these organisms. These bacteria have in-built mechanisms that find ways to resist treatment and transfer genetic materials to other bacteria (WHO, 2017). Although the percentage resistance exhibited by the isolates was low compared to other antibiotics screened, the results from this study indicate that carbapenem-resistant *Pseudomonas* spp. is present in the study area. Carbapenem-resistant *Pseudomonas* spp. has been reported from clinical settings in South Africa (Prinsloo, van Straten and Weldhagen 2008; Mudau *et al.* 2013; Mendelson and Matsoso 2015), however, this has not been reported from environmental samples. The surveillance of carbapenem resistance globally has limitations in terms of the lack of reliable data from regions such as Sub-Saharan Africa (Codjoe and Donkor 2018). In South Africa, there is more focus on clinical isolates as compared to environmental isolates, which is a global trend with regards to antibiotic resistance investigations (Ekwanzala *et al*. 2018).

A high number of *Pseudomonas* isolates displayed resistance against ampicillin (93%), cefixime (95%), polymyxin B (36%), and minocycline (14%). Cefixime is a third-generation cephalosporin (Garbis *et al*. 2007), whose resistance increases because of extended-spectrum *β*-lactamases (ESBL's) and carbapenemase activity (Livermore and Woodford 2006). Genes on large plasmids code ESBL's and these plasmids convey genes that bring about resistance against other antibiotics (Thenmozhi *et al*. 2014). As a result, broad antibiotic resistance extending over various classes of antibiotics is now a common attribute of ESBL producing organisms (Rawat and Nair 2010).

ESBL's limit the efficacy of *β*-lactams including extended-spectrum cephalosporins and thus it is associated with high morbidity and mortality. Susceptibility patterns of *Pseudomonas* isolates obtained in this study are similar to other studies which reported increased resistance against ampicillin (Odjadjare *et al*. 2012) and cotrimoxazole/trimethoprim-sulphamethoxazole (Allydice-Francis and Brown 2012). The tested isolates were susceptible to traditional antibiotics such as fluoroquinolones (ofloxacin, levofloxacin, and ciprofloxacin) and the polypeptide (colistin). Fluoroquinolones are widely prescribed as antimicrobial agents because of their spectrum of activity. Even though ciprofloxacin is an older, narrow-spectrum antibiotic, it is effective against Gram-negative bacteria such as *Pseudomonas* spp. (El Solh and Alhajhusain 2009).

**Table 4.5 Antibiotic susceptibility profile of *Aeromonas* spp and *Pseudomonas* spp**

| Antibiotic | % Resistance | |
|---|---|---|
| | *Aeromonas* spp. (*n*=59) | *Pseudomonas* spp. (*n* =53) |
| Polymyxin B | 56 | 36 |
| Ampicillin | 76 | 93 |
| Cefixime | 5 | 95 |
| Ofloxacin | 0 | 2 |
| Levofloxacin | 8 | 4 |
| Ceftazidime | 5 | 4 |
| Ciprofloxacin | 2 | 5 |
| Colistin | 42 | 7 |
| Minocycline | 3 | 14 |
| Meropenem | 8 | 21 |
| Imipenem | 8 | 7 |
| Trimethoprim-sulphamethoxazole | 100 | 100 |

### 4.3.4 Detection of antibiotic resistance genes in confirmed *Aeromonas* spp. and *Pseudomonas* spp. isolates

Resistance against *β*-lactams is primarily initiated by *β*-lactamases, which are responsible for hydrolysing the *β*-lactam ring and as a result inactivating the antibiotic (Delmani *et al.* 2017). The presence of the $bla_{TEM}$ genes in water environments could result in the distribution of the *β*-lactamase gene into the environment and increase the risk of the environment becoming a reservoir for ARG (Piotrowska, Kowalska and Popowska 2019). The $bla_{TEM}$ genes were detected in 33% of *Aeromonas* spp. and 22% of *Pseudomonas* spp. Similar results were reported in *Pseudomonas* spp. from the effluent of a penicillin production plant and in the surface water downstream (Li *et al.* 2009; Igbinosa *et al*. 2012b). *Aeromonas* spp. have been reported to harbour the $bla_{TEM}$ gene, but they are not normally the most common host of this gene (Narciso-da-Rocha *et al*. 2014). Although 77% of *Pseudomonas* and 74% of *Aeromonas* isolates were resistant to ampicillin, the *blaAmpC* gene was only detected in 3% and 8% of isolates of *Pseudomonas* and *Aeromonas* isolates, respectively. Additionally, the *AmpC*/*blaOXA* gene was found in only 4% of *Aeromonas* isolates. *Aeromonas* spp. is known to carry the *blaOXA* gene and are found mainly in isolates from aquatic environments (Narciso-da-Rocha *et al*. 2014). Although, phenotypically, resistance was high, the low prevalence of the genes coding for resistance in the isolates could be an indication that the resistance to antibiotics is a function of more than one gene or factor (Evangelista-Barreto *et al.* 2010; Piotrowska and Popowska 2015). It was previously reported that though the development of antibiotic resistance is normally associated with genetic changes by acquiring resistance genes or mutations, in some instances, the resistance can be achieved phenotypically (Li, Plésiat and Nikaido 2015). Therefore, resistance against these antibiotics might have originated from other molecular mechanisms of resistance against antibiotics, such as the multidrug efflux pumps (Abebe, Tegegne and Tibebu 2016). This could be interlinked with mechanisms such as membrane permeability barrier, enzymatic inactivation, or antibiotic target changes (Peterson and Kaur 2018). In this study, both *Pseudomonas* and *Aeromonas* showed resistance against colistin and *Pseudomonas* to polymyxin B as well. However, the corresponding *mcr-1* gene was not detected in any of the isolates using PCR. Polymyxins (colistin and polymyxin

B) are used as the last line of defence against Gram-negative bacterial infections (Paterson and Harris 2016).

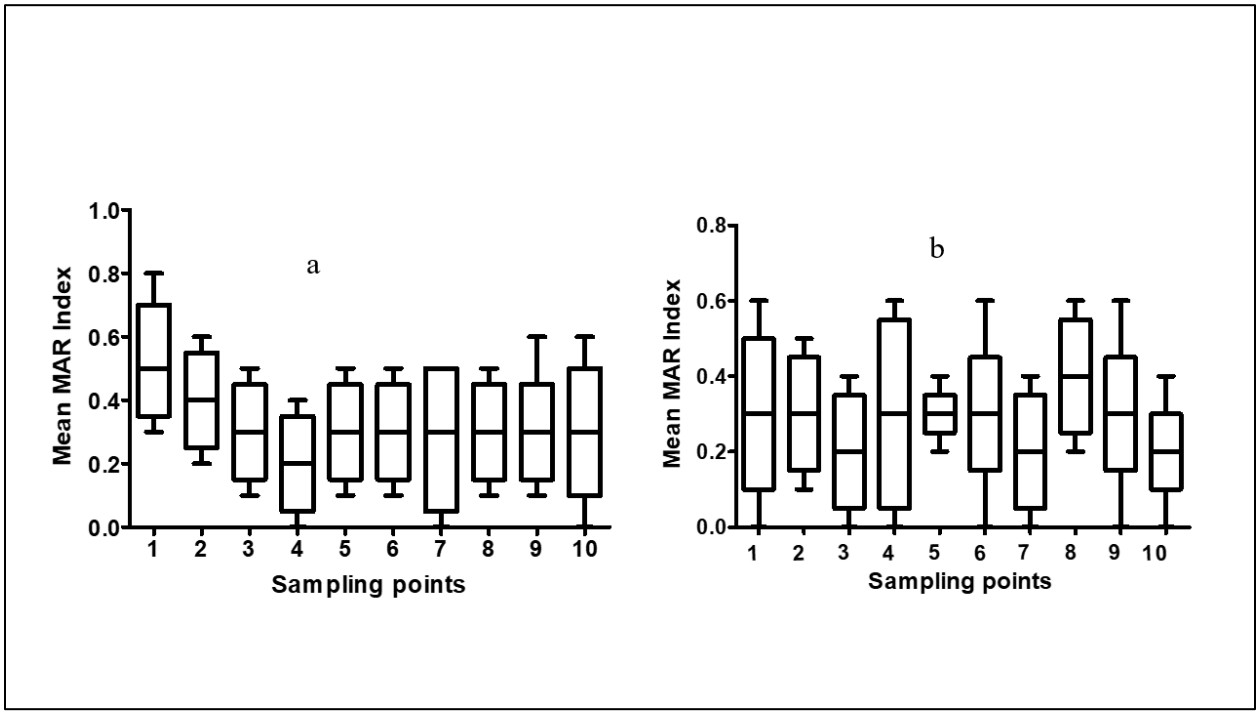


**Figure 4.1 Multi drug resistant profile of *Pseudomonas* spp. (*n*=53) (a) and *Aeromonas* spp. (*n*=59) (b) isolates at the different sampling points**

***Key: 1= Hospital upstream; 2= Hospital downstream; 3= Veterinary clinic upstream; 4= Veterinary clinic downstream; 5= Informal settlement upstream; 6= Informal settlement downstream; 7= WWTP influent; 8= WWTP effluent; 9= WWTP upstream; 10= WWTP downstream.**

### 4.3.5 Multiple antibiotic resistance indices of isolates from different sampling points

The MAR index for *Aeromonas* isolates ranged between 0.2 and 0.5 (Figure 4.1) showing that a large percentage of the isolates are in the high-risk category. Other studies showed MAR index values of *Aeromonas* spp. ranging from 0.4 to 0.8 (Paul *et al*. 2015). All isolates of *Aeromonas* and *Pseudomonas* had MAR indices above 0.2, which indicates that these bacteria were isolated from environments with high antibiotic selective pressure (Osundiya, Oladele and Oduyebo 2013).

There was no statistical difference in the MAR indices between the two organisms, which may be attributed to similar selective pressures for these bacteria. However, the MAR indices of isolates from the different sampling points showed a difference in the selective pressure. The highest index for *Pseudomonas* spp. was observed in isolates from both the upstream (0.5) and downstream (0.4) of the hospital. In respect of *Pseudomonas* spp., there was no difference between the indices in isolates from the other sampling points. This may be attributed to the intrinsic resistance in these bacteria. It has been reported that *Pseudomonas* spp. has the intrinsic ability to produce lactamases, the presence of the gene coding for this enzyme has been shown to result in resistance against several other antibiotics (Pang *et al*. 2019). So, the resistance at the sampling points may not necessarily be attributed to increased selective pressure from exposure to antibiotics but the natural ability of the bacterium.

The highest MAR index for *Aeromonas* spp. was observed in isolates from the WWTP effluents (0.4). WWTPs have been reported to be hotspots for antibiotic resistance due to the presence of a high concentration of antibiotics (Guo *et al*. 2017), microbial pathogens and ARG, which creates selective pressure for antibiotic resistance development. Therefore, it is likely that the MAR index for the isolates may be due to selective pressure within the WWTPs. However, it is worth noting that despite the high index in the effluent it had no direct influence on the antibiotic resistance profile of the isolates downstream of the WWTP. This could be attributed to the natural die-off of the bacteria when released into the surface water (WHO, 2016) or attachment to particulate matter in the river and therefore it is less frequently encountered in the water column (Oliver *et al*. 2007; Hassard *et al*. 2016).

### 4.3.6 Virulence gene detection in *Aeromonas* spp. and *Pseudomonas* spp. isolates

The pathogenicity of *Aeromonas* spp. is complex and involves various factors which act together or independently of each other at different stages of infection (Tomás 2012). The aerolysin gene (*aer*), which has haemolytic, cytotoxic and enterotoxin activity when expressed, was found in 31% of isolates. Aerolysin is a toxin that has pore-forming abilities (Cirauqui *et al.* 2017) and this was first detected in *A. hydrophila* (Podobnik, Kisovec and Anderluh 2017). Aerolysin plays an essential role in infections that are caused by *Aeromonas* spp. (Kingombe *et al.* 1999). The gene

for flagellin (*fla*) was detected in 25% of the isolates. The presence of flagella has been established as a virulence factor, polar flagella allow for swimming and lateral flagella enables swarming motility and both are linked with adsorption into the host's cell (Sen and Rodgers, 2004). Lateral flagella are known to help in adhesion to the epithelial cells of the human intestinal cells and are associated with dysenteric infections (Gavín *et al*. 2002). Flagella can be an important virulent factor since both types of flagella support biofilm formation and may contribute to repeated infections (Kirov, Castrisios and Shaw 2004). The two cytotonic enterotoxins (*ast* and *alt*) were not detected in the isolates, which was in accordance with a previous report by Nawaz *et al*. (2010).

The most prevalent putative gene detected from *Pseudomonas* spp. was *exoS* (26%), which is responsible for cytotoxin production. The detection of this gene corroborated with earlier reports, especially in *P. aeruginosa* isolates from wastewater (Igbinosa, Igbinosa and Okoh 2014*)*. Exotoxin A *(ToxA)* is an important virulence factor of clinical infections. It is cytotoxic, similar to the diphtheria toxin, and prevents protein biosynthesis, which leads to tissue and organ damage (Jenkins *et al*. 2004). The *toxA* gene was detected in 4% of the isolates. The *ecfX* gene encodes for extracytoplasmic function which is restricted to *P. aeruginosa* and is said to play a role in virulence and haem uptake (Lavenir *et al*. 2007). This gene was present in 18% of the isolates tested. The presence of *lasI* was detected in 4% of isolates, *lasI* is essential for transcription of genes for elastase (*lasB*), *lasA* protease, and alkaline protease, the three proteases that are associated with virulence (Aybey and Demirkan 2016). The probable presence of *Aeromonas* spp. and *Pseudomonas* spp. with multiple virulence factors may be unfavourable to the health of individuals that are exposed to the rivers as these organisms are potentially pathogenic.

### 4.3.7 Relationship between antibiotic resistance genes, resistance phenotype, and virulence genes in the *Aeromonas* spp. and *Pseudomonas* spp. isolates

For the convenience of further analysis of the positive or negative association of the isolates with RP, presence of ARG and VG, the identified strains of both bacteria were divided into five groups: *P. putida* (PG1), *P. aeruginosa* (PG2), *P. mendocina* (PG3), *P. alcaligenes* (PG4), *P. oleovorans* (PG5) for *Pseudomonas* spp. and *Aeromonas hydrophila/caviae* (AG1), *Aeromonas caviae* (AG2),

*Aeromonas hydrophila/caviae/sobria* (AG3), *Aeromonas* spp. (AG4), *Aeromonas veronii/sobria* (AG5) for *Aeromonas* spp.

The cross-tabulation data for the association groups, ARG, RP, and VG in *Pseudomonas* spp. (Table 4.6.) showed that the virulence gene *exo32* was significantly associated with antibiotic resistant gene $bla_{TEM}$ ($\chi^2$ =12.689, $p$<0.05, OR=13.333). Four *Pseudomonas* spp. groups were found to be significantly associated with one or more RPs but none with any ARG. VG *las32* was significantly associated with RP minocycline (Chi-squared valued ($\chi^2$) =20.755, $p$<0.05, Odds Ratio (OR)=1.666). PG1 showed significant association with RP ceftazidime ($\chi^2$ =5.984, $p$<0.05, OR=0.130) and imipenem ($\chi^2$ =4.362, $p$<0.05, OR=7.5). PG2 was significantly associated with RP colistin ($\chi^2$ =6.935, $p$<0.05, OR=15.333) and imipenem ($\chi^2$ =4.362, $p$<0.05, OR=7.5). PG3 and PG4 were found to be significantly associated with RP ceftazidime ($\chi^2$ =5.984, $p$<0.05, OR=0.130) and ampicillin ($\chi^2$ =21.000, $p$<0.05, OR=not calculated), respectively. The ARG *AmpC* and *blaOXA* were found to be significantly associated with RP meropenem and ampicillin ($\chi^2$ =4.074, $P$<0.05, OR=1.1). Studies have reported that the virulence and fitness of *Pseudomonas* spp. are altered during antibiotic resistance mutations especially in the case of multidrug efflux pump production and mutations (Geisinger and Isberg 2017; Li, Plésiat and Nikaido 2015). For instance, experimental data support the theory that hyperproduction of the resistance-nodulation-division class of pumps leads to a reduction in the fitness of the organism in the absence of antibiotic pressure (Sánchez, Alonso and Martinez 2002; Abdelraouf *et al*. 2011). The cross-tabulation results for antibiotic resistance and virulence from this study show that the development of resistance did not affect the virulence, as shown by the significant association observed. This is corroborated by data obtained with studies with *E. coli*, where Zhang *et al.* (2015b) showed that resistance was higher for pathogenic strains.

Cross-tabulation data for the association of groups, ARG, RP, and VG with each other shows that some *Aeromonas* spp. may arise as potential pathogens (Table 4.7.). The cross-tabulation data shows that VG *aer* was significantly associated with ARG $bla_{OXA}$ ($\chi^2$ =6.657, $P$<0.05, OR=53.0) and RP ceftazimide ($\chi^2$ =7.537, $p$<0.05, OR=0.259). VG *fla* was found to be significantly associated with ARG $bla_{AMP}$ ($\chi^2$ =6.397, $p$<0.05, OR=10.6), *sul1* ($\chi^2$ =10.319, $p$<0.05, OR=17.571) and *sul2* ($\chi^2$ =17.838, $p$<0.05, OR=25.0) as well as RP polymyxin B ($\chi^2$ =4.969, $p$<0.05,

OR=0.164). The VG *alt* showed significant association with RP levofloxacin ($\chi^2$ =4.902, $p$<0.05, OR=14.0) and minocycline ($\chi^2$ =14.484, $p$<0.05, OR=59.0). Only one group, AG4 was found significantly associated with RP meropenem ($\chi^2$ =3.993, $p$<0.05, OR=12.333). The ARG showed a significant association with RP imipenem ($\chi^2$ =4.253, $p$<0.05, OR=6.5), which is expected as ARG $bla_{OXA}$ is responsible for the resistance against RP imipenem in microorganisms. The results of the cross-tabulation show that there is a high probability that both the *Aeromonas* spp. and *Pseudomonas* spp. isolates are both pathogenic and resistant to these different classes of antibiotics. This could be attributed to the environment within which they were isolated, which could have created selective pressure.

**Table 4.6 Significant ($\chi^2$ $P < 0.05$) positive and negative associations between virulence gene, groups, antibiotic genes, and antibiotics in *Aeromonas* spp.**

| Virulence gene[1]/Group[1] /Antibiotic gene[1] | Antibiotic gene[1] | | | | | Antibiotics[1] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *blaOXA* | *blaAMPC* | *Sul1* | *Sul 2* | PolymyxinB | Levofloxacin | Imipenem | Ceftazimide | Minocycline | Meropenem |
| ***Aer*** | $\chi^2$6.657; OR 53(5-58) | ns | ns | ns | ns | ns | ns | $\chi^2$ 7.537; OR 0.259(0.167 -0.400 | ns | ns |
| ***Fla*** | ns | $\chi^2$ 6.397; OR 10.6(1.218 -92.268) | $\chi^2$ 10.319; OR 17.571(1.943- 158.935) | $\chi^2$ 17.838; OR 25(3.815 -163.86 | $\chi^2$ 4.969; OR 0.164(0.029-0.935 | ns | ns | ns | ns | ns |
| ***Alt*** | ns | ns | ns | ns | ns | $\chi^2$ 4.902; OR 14(0.732-267.909 | ns | ns | $\chi^2$ 14.484; OR 59(1.961-1775.559 | ns |
| **AG4** | ns | ns | ns | ns | ns | ns | ns | ns | ns | $\chi^2$ 3.993; OR 12.333(0.607-250.508 |
| ***blaOXA*** | ns | ns | ns | ns | ns | ns | $\chi^2$ 4.253; OR 6.5(0.899-47.018 | ns | ns | ns |

$\chi^2$ represents the Chi-square value, OR is odds ratio using a 95% confidence interval while ns is not significant. [1]Virulence gene, phylogenetic groups, antibiotic genes, and antibiotics showing no significant associations with each other are not shown in the table. AG4 represents *Aeromonas* spp.

**Table 4.7 Significant ($\chi^2$ $P < 0.05$) positive and negative associations between virulence gene, groups, antibiotic genes, and antibiotics in *Pseudomonas* spp.**

| **Virulence gene[1]/ Group[1]/Antibiotic gene[1]** | **Antibiotic gene[1]** | **Antibiotics[1]** | | | | | |
|---|---|---|---|---|---|---|---|
| | *blaTEM* | Ceftazidime | Colistin | Imipenem | Ampicillin | Minocycline | Meropenem |
| ***exoS*** | $\chi^2$ 12.689; OR 13.333(2.612-68.054) | ns | ns | ns | ns | ns | ns |
| ***lasI*** | ns | ns | ns | ns | ns | $\chi^2$ 20.755; OR 1.666(0.815-3.409 | ns |
| **PG1** | ns | $\chi^2$ 5.984; OR 0.130(0.065-0.259) | v | $\chi^2$ 4.362; OR 7.5(0.885-63.559) | ns | ns | ns |
| **PG2** | ns | ns | $\chi^2$ 6.935; OR 15.333(1.201-195.739) | $\chi^2$ 4.362; OR 7.5(0.885-63.559) | ns | ns | ns |

| **Virulence gene[1]/ Group[1]/Antibiotic gene[1]** | **Antibiotic gene[1]** | **Antibiotics[1]** | | | | | |
|---|---|---|---|---|---|---|---|
| | *blaTEM* | Ceftazidime | Colistin | Imipenem | Ampicillin | Minocycline | Meropenem |
| **PG3** | ns | $\chi^2$ 5.984; OR 0.130(0.065-0.259) | ns | ns | ns | ns | ns |
| **PG4** | ns | ns | ns | ns | $\chi^2$ 21.000; OR (Not Calculated) | ns | ns |
| ***AmpC*** | ns | ns | ns | ns | ns | ns | $\chi^2$ 4.074; OR 1.1(0.913-1.326) |
| ***blaOXA*** | ns | ns | ns | ns | ns | ns | $\chi^2$ 4.074; OR 1.1(0.913-1.326) |

$\chi^2$ represents the Chi-square value, OR is odds ratio using a 95% confidence interval while ns is not significant. [1]Virulence gene, phylogenetic groups, antibiotic genes, and antibiotics showing no significant associations with each other are not shown. PG1 represents *P. putida*, PG2 – *P. aeruginosa*, PG3- *P. mendocina*, PG4 - *P. alcaligenes*.

## 4.4 Conclusions

The results of this study confirm the presence and persistence of potential opportunistic pathogens belonging to *Pseudomonas* spp. and *Aeromonas* spp. in wastewater and surface water environments. Despite the removal of these potential pathogens during wastewater treatment, it was observed that the final effluents still contain high concentrations of these bacteria. However, this did not have a significant impact on the concentrations downstream of the treatment plant. Samples from downstream of a hospital and veterinary clinic showed that these sites might significantly impact concentrations of bacteria in surface water. *Aeromonas* isolates recovered from both wastewater and surface water displayed high resistance against ampicillin and had higher MAR indices especially close to the hospital. *Pseudomonas* isolates on the other hand exhibited low resistance against carbapenems but very high resistance against the third-generation cephalosporins, cefixime. Antibiotic resistance genes, *sul1/2*, $bla_{TEM,}$ and $bla_{AmpC}$ were prevalent in both *Aeromonas* spp. and *Pseudomonas* spp**.** whilst $bla_{AmpC}$ and $bla_{OXA}$ were detected only in *Aeromonas*. The VG *aer* and *fla* were detected in *Aeromonas* spp**.** while *exoS, toxA, ecfX,* and *lasI* were detected in *Pseudomonas* spp**.** The positive and significant association between the groups (ARG, RP, and VG) indicates that the bacterial isolates can be resistant to antibiotics and can also be pathogenic. This is of concern as surface water samples were collected from areas, which were constantly accessed by animal and human populations dwelling in nearby informal settlements. This is a major risk in a province known for a high population of immunocompromised individuals due to the high rate of tuberculosis and HIV infections.

# CHAPTER 5: Detection of Multi-Drug Resistant Environmental Isolates of *Acinetobacter* and *Stenotrophomonas maltophilia*: A Possible Threat for Community Acquired Infections?

**Govender, R**., Amoah, I. D., Kumari, S., Bux, F. and Stenström, T. A. 2020. Detection of multidrug-resistant environmental isolates of *Acinetobacter* and *Stenotrophomonas maltophilia*: a possible threat for community-acquired infections? ***Journal of Environmental Science and Health, Part A***: 1-13.

## 5.1 Introduction

The emergence and spread of antibiotic-resistant bacteria and genes in the aquatic environment are one of the key public health concerns globally (Kraemer, Ramachandran, and Perron 2019). In developing countries, such as South Africa where waterborne outbreaks occur frequently, there is the need for enhanced measures to reduce the associated risks of these outbreaks. Some of these measures could be improved monitoring of the occurrence and concentration of contaminants in water. Infection with waterborne diseases is mainly due to either direct, accidental or intentional exposure, where lack of potable water or use of contaminated water is a major route of transmission (Genthe *et al.* 2013). Indirect exposure could be through the consumption of food prepared with contaminated water. Direct exposure is however central for the organisms that are part of this study: *Acinetobacter* spp. (Rebic *et al.* 2018) and *Stenotrophomonas maltophilia* (Adegoke, Stenström and Okoh 2017), which can be life-threatening especially among immunocompromised individuals and other vulnerable groups. Due to the health challenges associated with antibiotic-resistant bacterial infections, the South African Antimicrobial Resistance Strategy framework (Departments of Health and Agriculture, Forestry and Fisheries, 2019) was developed to combat the spread of antibiotic resistance. The current study focuses on two of the most important bacteria associated with nosocomial infections; *Acinetobacter* spp. and *S. maltophilia.*

*S. maltophilia* is an emerging pathogen that is commonly found in hospital environments (Brooke 2012; Zhao *et al.* 2015; Singhal, Kaur and Gautam 2017), with clinical cases linked to hospital equipment and medical solutions (Dancer 2014). This organism has biofilm-forming capabilities in the hospital environment as well as in river water, wastewater treatment works,

and plant rhizospheres (Singhal, Kaur and Gautam 2017). *S. maltophilia* is an opportunistic pathogen responsible for life-threatening infections in both immunocompromised as well as immunocompetent individuals. It has been linked to and caused infections of the respiratory tract (Chawla, Vishwanath and Gupta 2014), bloodstream (Garazi *et al.* 2012), bone and joint infections (Al-Anazi and Al-Jasser 2014), urinary tract (Kumar *et al.* 2015), and meningitis (Falagas *et al.* 2009; Looney, Narita and Mühlemann 2009). This pathogen has been implicated in bacteraemia responsible for death in non-burned and burned patients; with a mortality rate of 10-69% and 30.7% respectively (Tsai *et al.* 2006). The incidence of *S. maltophilia in* hospital-acquired infections in South Africa has been reported in a few studies, these include cystic fibrosis infected patients (Mhlongo, Essack and Govinden 2015), intra-abdominal infections (Brink *et al.* 2012), bloodstream infections (Bisiwe *et al.* 2015; McKay and Bamford 2015) and ventriculostomy infections (Motloba and Ngqandu 2015). In a recent study by Muchesa *et al*. (2017), *S. maltophilia* was found to be co-existing with free-living amoeba in South African hospital water distribution systems, and this further highlights the risk to immune-compromised patients.

Only a few studies have reported *S. maltophilia* in environmental samples, such as wastewater, surface water (Adjidé *et al.* 2010), and plant rhizospheres (Berg, Eberl and Hartmann, 2005). MDR strains are increasingly being reported, resulting in enhanced mortality and morbidity rates (Jeon *et al.* 2016). Although the drug trimethoprim-sulfamethoxazole remains the dominant treatment of choice, there is an increased emergence of resistance against this drug (Li and Li, 2017).

Of the more than 30 species of the genus *Acinetobacter*, *A. baumannii* is considered to be most clinically significant, due to its ability to cause serious MDR infections in humans (Dijkshoorn and van der Toorn, 1992). Infections caused by *Acinetobacter* species include bacteraemia (Fishbain and Peleg, 2010), pneumonia (Yang *et al.* 2013), meningitis (Kim *et al.* 2009), catheter-related bloodstream infections (Wisplinghoff *et al.* 2012), intra-abdominal infections (Sartelli, 2010), urinary tract infections (Sartelli, 2010), and skin and soft tissue infections (Vâţă *et al.* 2012). The bacteria commonly infect critically ill, immunosuppressed patients and has been associated with a mortality of between 8% and 40% (Fournier, Richet and Weinstein, 2006; Eliopoulos, Maragakis and Perl, 2008; Peleg, Seifert and Paterson 2008). *Acinetobacter* poses an increasing health problem resulting in infections with limited to no antibiotic

therapeutic options available. WHO has published a list of antibiotic-resistant priority pathogens that pose a major threat to humans. This list is divided into categories that are determined by the urgency of the need for new antibiotics. *Acinetobacter baumannii* is a priority 1 organism and is included in the most critical group (WHO, 2017). Additionally, this pathogen is part of the ESKAPE list of pathogens (Alqahtani, 2017). The ESKAPE list consists of six nosocomial pathogens with growing multidrug resistance and virulence: *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* spp. (Mulani *et al*. 2019). These pathogens have been reported to be responsible for most nosocomial infections due to their ability to 'escape' the effect of antimicrobial agents (Mulani *et al*. 2019; Navidinia, 2016).

For both, *Acinetobacter,* and *S. maltophilia* (Hotta *et al*. 2014; Alqahtani, 2017), most reported infections occur among hospitalized patients, but patients in the community settings may likewise be vulnerable. Although, *Acinetobacter* and *S. maltophilia* species have been isolated from various environmental sources it is unclear whether these are 'true environmental' isolates or are a result of contamination from other sources, such as clinical settings (Eveillard *et al.* 2013; Antunes Visca and Towner 2014). Community-acquired infection is often common among immunocompromised individuals, whilst having an underlying medical condition. With an HIV prevalence of 20% as of 2018, South Africa has a high proportion of immunocompromised individuals, therefore the risks of community-acquired infections with these pathogens are high (UNAIDS, 2019). The key focus areas of this chapter was to determine the prevalence of *Acinetobacter* spp. and *S. maltophilia* in water matrices and to determine the community risks associated with their presence.

## 5.2 Methodology

### 5.2.1 Description of the study area

For this study, surface water samples were collected approximately 500 m upstream and downstream from the respective four main sites along the Umhlangane River, thus summing up to eight sampling points (**Chapter 3: Figure 3.2., Table 3.1.**). Plants and sediments were also collected for isolating *S. maltophilia* due to the reported high concentration of this pathogen within these environmental niches **(**Bendadeche, Hamad and Ayad 2019**;** An and

Berg, 2018). The study area has been described in detail in **Chapter 3: Section 3.2.** of this thesis.

### 5.2.2 Sample collection

Surface water (*n*=48)/ wastewater (*n*=12) samples, sediment (*n*=16), and plant samples (*n*=16) were collected and stored as described in **Chapter 3: Section 3.3.1** and **3.3.2,** respectively.

### 5.2.3 Identification of the bacterial isolates

#### 5.2.3.1 Quantification and isolation of presumptive *S. maltophilia* and *Acinetobacter* isolates

The isolation of target bacteria from water, sediment, and plant samples was described **in Chapter 3: Section 3.4.1., 3.4.2. and 3.4.3.** respectively. The selective media used were *Stenotrophomonas* selective agar base (Himedia, India) with Vancomycin Imipenem Amphotericin B supplement (Himedia, India) and CHROMagar *Acinetobacter* (CHROMagar, France) with a multiple drug-resistant supplement (CR 102) (**Chapter 3: Table 3.3**). Both media were prepared according to the manufacturer's instructions. After incubation, as per **Chapter 3: Table 3.3**., the presumptive colonies were enumerated, characterised, and recorded. The results were expressed as CFU/mL of water and CFU/g for plant and sediments.

Presumptive isolates with a typical appearance on their respective medium (red for *Acinetobacter,* cream colonies for *S. maltophilia*) were purified as described in **Chapter 3: Section 3.6**. The presumptive isolates were Gram-stained as described in **Chapter 3: Section 3.7.1.1** and the Gram-negative isolates were checked for their oxidase reaction as described in **Chapter 3: Section 3.7.1.2.** In addition to the above, a catalase test was performed on the isolates as described in **Chapter 3: Section 3.7.1.3**. The identity of the presumptive isolates was confirmed by PCR and was further validated by MALDI-TOF MS (**Chapter 4: Section 4.2.5**).

#### 5.2.3.2 Molecular confirmation of *S. maltophilia* and *Acinetobacter* isolates

PCR was used to amplify and detect the presence of specific conserved sequences in presumptive *S. maltophilia* and *Acinetobacter* isolates using genus-specific primers (Inqaba Biotech, SA) as listed in Table 5.1. DNA was extracted using the boiling method (Jyoti *et al*. 2011) as described in **Chapter 3: Section 3.8.1**. Extracted DNA was amplified in a Bio-Rad,

T100 thermal cycler (Bio-Rad, USA) as described in **Chapter 3: Section 3.8.2**. The translation elongation factor P gene (*efp*) was targeted for the genus-specific identification of *Acinetobacter*. The thermal cycling conditions included an initial denaturation for 4 min at 94°C, and 35 cycles of denaturation at 95°C for 45 s, annealing at 52°C for 45 s, and extension at 72°C for 90 s and a final extension at 72°C for 10 min. *Acinetobacter baumannii,* ATCC 19606 was used as a positive control for the PCR assay. The identification of *S. maltophilia* was based on a 278 bp fragment of the 23S rRNA gene. The 23S rRNA gene was selected because there is higher variability in this region among species of the *Stenotrophomonas* genus in comparison with the 16S rRNA gene (Gallo *et al.* 2013). The amplifications were performed with an initial denaturation step at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 68°C for 45s and extension at 72°C for 45s, with a final extension at 72°C for 10 min. *Stenotrophomonas maltophilia* (ATCC 13637) was used as a positive control in the assay. PCR reactions were performed in 25 µL volumes that constituted 50 ng/µL of the template DNA, 0.4 µM of each primer set, 1x Maxima Hotstart PCR master mix (Thermo Scientific, USA), and nuclease-free water (Thermo Scientific, USA).

**Table 5.1 Primers used in this study**

| Target/ Primer name | Sequence (5'–3') | Amplicon size (bp) | Reference |
|---|---|---|---|
| *efp (Acinetobacter* spp.) | AGCCAGGCCTTAAGGTCATG<br>GCCAGAAGTATCACCACGTA | 422 | (Anbazhagan *et al.* 2011) |
| *23sRNA (S. maltophilia)* | GCTGGATTGGTTCTAGGAAAACGC<br>ACGCAGTCACTCCTTGCG | 278 | (Gallo *et al.* 2013) |
| $bla_{OXA23}$ | GATCGGATTGGAGAACCAGA<br>ATTTCTGACCGCATTTCCAT | 501 | (Bagheri Josheghani *et al.* 2015) |
| $bla_{OXA51}$ | TAATGCTTTGATCGGCCTTG<br>TGGATTGCACTTCATCTTGG | 353 | (Bagheri Josheghani *et al.* 2015) |
| $sme_{ABC}$ | ACCGCCCAGCTTTCATACA<br>GACATGGCCTACCAGGAACA | 60 | (Herrera-Heredia *et al.* 2017) |
| $sme_{DEF}$ | TCGTCCAGGCTGACATTCA<br>AACGCGGATCGTGATATC | 62 | (Herrera-Heredia *et al.* 2017) |

#### 5.2.3.3. Electrophoresis of PCR products

The amplified PCR products were visualized with agarose gel electrophoresis for the specific product as described in detail in **Chapter 3: Section 3.8.3**.

### 5.2.4 MALDI-TOF MS identification

The PCR-identified isolates were further confirmed with MALDI-TOF MS. This was described in detail in **Chapter 4: Section 4.2.5**.

### 5.2.5 Antibiotic resistance profiling

Antibiotic resistance testing was performed as per the method described in **Chapter 4: Section 4.2.6**.

### 5.2.6 Multiple antibiotic resistance index

The MAR index was determined for all organisms that displayed multiple drug resistance. MAR index calculations were further described in **Chapter 4: Section 4.2.7**.

### 5.2.7 PCR detection of antibiotic resistance and efflux genes in *S. maltophilia* and *Acinetobacter* and virulence genes in *Acinetobacter* species

The DNA extracts of *S. maltophilia* and *Acinetobacter* that were extracted previously as described in **Chapter 3: Section 3.8.1**., were used for the screening of isolates for antibiotic resistance genes, VG, and efflux genes (*S. maltophilia*). The primers used for the detection of antibiotic-resistant and efflux genes were listed in **Chapter 4: Table 4.1.** (*bla$_{TEM}$*, *bla$_{AmpC}$*, *mcr-1*, *sul1, sul2, sul3)* and **Table 5.1**. *S. maltophilia* was screened for efflux genes (*sme$_{ABC}$*; *sme$_{DEF}$*) following the method used by Herrera-Heredia *et al.* (2017). The PCR reaction was carried out as described in **Chapter 3: Section 3.8.2.** PCR was initiated by an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 60 s, extension at 68°C for 60 s and final extension at 72°C for 5 min (Herrera-Heredia *et al.* 2017). Gel electrophoresis was carried out as described in **Chapter 3: Section 3.8.3.** *Acinetobacter* spp. was screened for VG (*ompA*, *epsA*, *cnf1*, *csgA*, *cvc,* and *iutA)* (Tayabali *et al*. 2012; Al-Kadmy *et al*. 2018). All PCR reactions were done in a volume of 25 $\mu$L using the conditions described previously (**Chapter 3: Section 3.8.2.**).

### 5.2.8 Statistical analysis

GraphPad Prism 5.0 was used for data analysis. The two-tailed t-test and two-way ANOVA were used to determine the *p*-values, where the predictive values were calculated at a 95% confidence level.

## 5.3 Results and discussion

### 5.3.1 Detection and quantification of presumptive *Acinetobacter* and *S. maltophilia* isolates

*Acinetobacter* and *S. maltophilia* were found in the water samples from across the different sampling points, at varying prevalence and concentrations. The concentration of *Acinetobacter* varied from 2.0 – 2.6 $\log_{10}$ CFU/mL (Table 5.2.), with the highest concentration (2,6±1,2 $\log_{10}$ CFU/mL) reported upstream of the WWTP effluent discharge point. The difference in *Acinetobacter* concentrations in the water samples from the various sampling points was not statistically significant ($p \geq 0.05$). The occurrence of *Acinetobacter* spp. in non-clinical settings, such as surface water, has been reported in South Africa (Stenström, Okoh and Adegoke, 2016), and other countries (Silva *et al.* 2016; Higgins *et al.* 2018; Proia *et al.* 2018; Wang, Wang and Yang 2018, Gao *et al.* 2020). Despite reports of *Acinetobacter* spp. in municipal wastewater and hospital wastewater (Silva *et al.* 2016; Proia *et al.* 2018; Gao *et al.* 2020) and the general reports of contamination of surface water from these sources, (Emmanuel *et al.* 2002; Al-Gheethi *et al.* 2018) the findings in this study did not show impact from any of these sources. No significant difference ($p \geq 0.05$) was observed in the concentration of *Acinetobacter* which ranged from 2.0±1.5 to 2.6±1.2 ($\log_{10}$ CFU/mL), at the upstream and downstream sampling points.

*S. maltophilia* was detected less frequently in the water column samples, with only three sampling sites showing the presence of this pathogen. These sites were upstream of the hospital (Site 1) and upstream and downstream of the WWTP (Site 2) (Table 3.1). Despite the low prevalence of *S. maltophilia* in the water samples, the highest concentration was recorded for this pathogen at the sampling point upstream of the wastewater discharge point. A statistically significant difference was observed when comparing the concentration at the wastewater discharge point to the downstream sampling point. *S. maltophilia* has been reported in

wastewater (Chang *et al*.2005; Adjidé *et al*. 2010), river water samples (Nakatsu *et al.* 1995), lake (Rivas *et al.* 2009), and drinking water (Silbaq 2009; Simões *et al.* 2007). Therefore, the findings in this study are corroborated by other reports. However, despite the reports on the occurrence of this potential pathogen in wastewater, findings in this study show that the wastewater effluent discharge is not the major contributory factor to the bacterial contamination in the river. This could be from surface runoff (Sasakova *et al.* 2018). It is hypothesised that the higher concentration of *S. maltophilia* in comparison to *Acinetobacter*, at site 1 and site 2 (Table 3.1.), in the water column (Table 3.1.), could be due to the presence of a higher concentration of *S. maltophilia* in the rhizosphere of the grasses that grow along the banks of the river. This could result in the dissemination of the bacteria into the water column when disturbed. A previous study by Adegoke and Okoh (2015) reported that 96% of S. *maltophilia* isolates were isolated from the grass-root rhizosphere. However, this hypothesis warrants further investigation.

Comparatively, high concentrations of *S. maltophilia* were found in the sediments (3.8 – 5.0 $\log_{10}$ CFU/g) and plant roots (3.6 – 4.4 $\log_{10}$ CFU/g). With the occurrence of the pathogen in plant rhizosphere and river sediment samples from all the sites studied. The findings in this study have been corroborated by other findings in the literature, where S. *maltophilia* was found to be associated with roots of different plants (Pereira *et al.* 2011; Zhu *et al.* 2012; Dawwam *et al.* 2013; Adegoke and Okoh 2015; Majeed *et al.* 2015). Plant roots are known to be microbial 'hot spots' where bacteria flourish (Berg, Roskot and Smalla 1999; Dungan, Yates and Frankenberger, 2003); Berg, Eberl and Hartmann, 2005; An and Berg, 2018 Bendadeche, Hamed and Ayad 2019). It has been reported that *S. maltophilia* promotes plant growth and it has antagonistic activity against plant pathogens (fungal and bacterial infections) due to chitinolytic activities (Zhang and Yuen 2000) and the production of phytohormones (Peralta *et al.* 2012).

**Table 5.2 Log CFU/mL of *Acinetobacter* and *S. maltophilia* in the water, sediments and plant rhizosphere samples**

| **Target organism** | ***Acinetobacter*** | **log values ± SD** ***S. maltophilia*** | |
|---|---|---|---|
| **Sample Point** | **Water** | **Water** | **Sediments** | **Plant rhizosphere** |
| **Hospital Upstream** | 2.2±1.4 | 3.2±0.1 | 3.8±0.1 | 4.0±0.8 |
| **Hospital Downstream** | 2.2±1.3 | 0 | 5.0±0.1 | 3.6±0.1 |
| **WWTP Upstream** | 2.6±1.2 | 4.1±1.0 | 4.3±0.8 | 4.2±0.6 |
| **WWTP Downstream** | 2.4±1.1 | 2.7±0.3 | 4.0±0.1 | 3.9±0.9 |
| **Informal Settlement Upstream** | 2.2±0.9 | 0 | 4.2±0 | 4.4±0.8 |
| **Informal Settlement Downstream** | 2.5±1.5 | 0 | 4.2±0 | 4.4±0.8 |
| **Veterinary clinic Upstream** | 2.0±1.5 | 0 | 4.0±0.2 | 4.1±0.8 |
| **Veterinary clinic Downstream** | 2.1±1.1 | 0 | 4.5±0.4 | 4.4±1.0 |

### 5.3.2 Identification of the isolates

The MALDI-TOF MS analysis (Table 5.3) showed that out of the 38 *Acinetobacter* isolates positively verified by PCR, 61% of the isolates were unknown *Acinetobacter* spp., 3% were *Acinetobacter haemolyticus,* 26% belonged to the Acb complex and 5% each were *Acinetobacter johnsonii* and *Acinetobacter lwoffi*. The Acb complex is made up of four *Acinetobacter* species which are *A. calcoaceticus (*previously known as *Acinetobacter* genospecies, *A. baumannii (*previously known as genospecies, *Acinetobacter pittii (*formerly known as genomic species, and *Acinetobacter nosocomialis* (known as genomic species 13TU) which are closely related and therefore difficult to distinguish them phenotypically (Nemec *et al*. 2015). The Acb complex is of high priority as a hospital-acquired pathogen and is often MDR (Fitzpatrick *et al.* 2015) which poses a challenge to infection management practices in the hospital environment (Chen *et al.* 2018). All species that are part of this complex have been previously associated with human infections apart from *A. calcoaceticus* which is considered to be an environmental isolate (Peleg, Seifert and Paterson 2008; Fitzpatrick *et al.* 2015). The remaining isolates in addition to this complex were identified by MALDI-TOF MS; **(***Acinetobacter haemolyticus, Acinetobacter johnsonii,* and *Acinetobacter lwoffi)* have been reported to be pathogens and have been responsible for causing various infections (Wong *et al.* 2017). For instance, *Acinetobacter lwoffi* which made up 5% of the *Acinetobacter* isolated has been reported to be a potential opportunistic pathogen in the immunocompromised and is responsible for infections such as septicaemia, acute gastritis, pneumonia, meningitis, urinary tract infections, and skin and wound infections (Regalado, Martin and Antony 2009). *Acinetobacter johnsonii*, which was 5% of the total isolates has been implicated in catheter-related bloodstream infections (Seifert *et al*. 1993). *A. haemolyticus* constituted 3% of the isolates and has previously been reported to be present in abscesses, wound infections and has resulted in septicaemia in the clinical setting (Tripathi, Gajbhiye and Agarwal 2014). A similar study in the Eastern Cape province of South Africa reported the presence of only *A. haemolyticus* and *A. calcoaceticus* in surface water *(*Stenström, Okoh and Adegoke, 2016). The findings in the current study, therefore shows that there are more species of this genus occurring in the environment. All *S. maltophilia* isolates positively identified by PCR were also validated by MALDI-TOF MS and represented the successful isolation of 25 isolates of *S. maltophilia* from environmental samples.

**Table 5.3 Confirmation of *Acinetobacter* and *S. maltophilia* isolates using PCR and MALDI-TOF MS**

| **Method** | ***Acinetobacter* spp.** | | ***S. maltophilia*** | |
|---|---|---|---|---|
| **PCR** | *n*=38 | | *n*=25 | |
| **MALDI-TOF MS** | *n*=38 | % | *n*=25 | % |
| | *Acinetobacter* sp. | 61 | *S. maltophilia* | 100 |
| | *Acinetobacter haemolyticus* | 3 | | |
| | *Acinetobacter baumannii* complex | 26 | | |
| | *Acinetobacter johnsonii* | 5 | | |
| | *Acinetobacter lwoffi* | 5 | | |

### 5.3.3 Antimicrobial resistance profiling

All isolates were tested for antibiotic susceptibility against 12 antibiotics (Table 5.4). Out of the 25 *S. maltophilia* isolates 100% showed resistance towards the sulphonamides (trimethoprim-sulphamethoxazole) which is the class of antibiotics of choice for treating *S. maltophilia* infections (Wang *et al.* 2014) (Table 5.4). Increasing resistance against trimethoprim-sulphamethoxazole has been reported in clinical isolates of *S. maltophilia* (Wang *et al.* 2016). Resistance against trimethoprim-sulfamethoxazole (26.1%) was also reported in isolates from plant rhizospheres (Adegoke and Okoh 2015). This suggests that in the instance of an outbreak of *S. maltophilia* infection, the recommended drug for treatment, trimethoprim-sulphamethoxazole, may not be effective. Alternatively, based on this study, antibiotics such as ofloxacin, minocycline, and levofloxacin could be effective against *S. maltophilia* since isolates showed susceptibility to these antibiotics. Previous studies showed the effectiveness

of the fluoroquinolones (which includes ofloxacin and minocycline), levofloxacin, and ciprofloxacin against clinical isolates of *S. maltophilia* (Wu *et al.* 2013; Ko *et al.* 2019).

In addition, *S. maltophilia* isolates showed resistance against the carbapenems (imipenem [100%] and meropenem [100%]) and third-generation cephalosporin's (cefixime [100%] and ceftazidime [80%]). Resistance against carbapenems has been reported to be intrinsic in *S. maltophilia* and thus carbapenems are not considered suitable for treating infections caused by *S. maltophilia* (Chang *et al*. 2015; Meletis, 2016). Due to the increased resistance of *S. maltophilia* against available antibiotics (trimethoprim-sulphamethoxazole), Chang *et al.* (2015) and Cho *et al.* (2015) suggested a combination of antibiotics to increase treatment efficiency.

Among *Acinetobacter* species, *A. baumannii* is the most important member associated with hospital-acquired infections worldwide (Lin and Lan, 2015). Many reports have shown that *A. baumannii* rapidly develops resistance against antimicrobials, and multidrug-resistant strains have been isolated from clinical samples (McConnell, Actis and Pachón 2013). In this study, *Acinetobacter* isolates were found to be resistant to trimethoprim-sulphamethoxazole (96%), polymyxins (*i.e.*, polymyxin B [86%], and colistin [42%]). Polymyxins are prescribed as last-line therapeutic agents for *A. baumannii* (Cheah *et al.* 2016). Carbapenems are the first choice of treatment for *Acinetobacter* infections; in this study, 4% of isolates were resistant to imipenem and 19% to meropenem. Polymyxins are prescribed as last-line therapeutic agents for *A. baumannii* isolates which are resistant to commonly used antibiotics such as carbapenems. (Cheah *et al.* 2016). Clinical studies in other regions have shown that *A. baumannii* was sensitive to polymyxins (100% of isolates in Algeria, 70.9% in Saudi Arabia, 92.5% in Kuwait, and 95% in Egypt (Al-Agamy *et al*. 2014). Colistin resistance in *A. baumannii* has been reported as uncommon, however, there have been cases of outbreaks in Italy, Korea, and Spain (Agodi *et al*. 2014; Lee *et al*. 2014; Pournaras *et al*. 2014). Therefore, the 42% resistance against colistin in *Acinetobacter* isolates from the water column in this study could be a major concern and can pose serious health risks for populations that rely on this river.

**Table 5.4 Antimicrobial susceptibility testing**

| **Antibiotic** | **% Resistance** | |
|---|---|---|
| | *Acinetobacter* | *S. maltophilia* |
| **Trimethoprim-sulphamethoxazole** | 96 | 100 |
| **Polymyxin b** | 86 | 36 |
| **Ofloxacin** | 4 | 0 |
| **Levofloxacin** | 0 | 4 |
| **Ceftazidime** | 8 | 80 |
| **Ciprofloxacin** | 4 | 8 |
| **Colistin** | 42 | 12 |
| **Minocycline** | 2 | 0 |
| **Meropenem** | 19 | 100 |
| **Imipenem** | 4 | 100 |
| **Ampicillin** | 35 | 100 |
| **Cefixime** | 54 | 100 |

### 5.3.4 Multiple antibiotic resistance index of the isolates

Both, *S. maltophilia* and *Acinetobacter* isolates displayed MDR (Figure 5.1.) where 100% of *S. maltophilia* isolates (irrespective of the sample matrix) and 78.9% of *Acinetobacter* isolates had MAR indices which were greater than 0.2. The MAR indices recorded in this study, therefore shows that these isolates were from areas with high levels of antibiotic contamination (Osundiya, Oladele and Oduyebo 2013). Comparatively, the MAR indices for the *S. maltophilia* isolates showed that the selection pressure for the antibiotics part of this study was higher than that for *Acinetobacter*. This is based not only on the percentage of these isolates with MAR above 0.2 but due to *S. maltophilia* isolates from the water samples having a MAR index from 0.6 to 0.8 (Figure 5.1; Chart A). The MAR indices for the *Acinetobacter* spp. isolated in this study are similar to the study by Stenström, Okoh and Adegoke, 2016, where MAR indices between 0.22 - 0.67 were reported in isolates from surface water in the Eastern Cape province of South Africa.

The results from this study show that *S. maltophilia* isolates downstream of the hospital and upstream of the WWTP had higher MAR indices of 0.8 and 0.7, respectively.

Similarly, some of the highest MAR indices for the *Acinetobacter* isolates were recorded in isolates from the hospital downstream samples (0.4) and upstream of the WWTP (0.5). The sampling point upstream of the WWTP is located downstream of the hospital sampling point (Table 3.1.). Therefore, it is likely that the heavy antibiotic use in the hospital to treat infections may have contributed to the MAR indices recorded at these two sampling points. The occurrence and persistence of antibiotic resistance within the hospital setting have been reported previously (Wang *et al*. 2018; Proia *et al.* 2018), thus the findings in this study is supported.

*S. maltophilia* isolates from the river sediment and plant rhizosphere samples had lower MAR indices than the water isolates, although MDR was less frequent in the water isolates (Figure 5.1., Chart B). Antibiotics have been reported to accumulate in sediments (Hu and Zhou 2012; Ye *et al*. 2007), therefore the selection pressure for the *S. maltophilia* isolates taken from the river sediments was expected to have higher MAR indices. However, the results of this study suggest that the antibiotic resistance may have developed outside this environment, or that the concentration of the antibiotics commonly used for the treatment of *S.*

*maltophilia* was not high in the sediments in this study area to result in antibiotic resistance development.

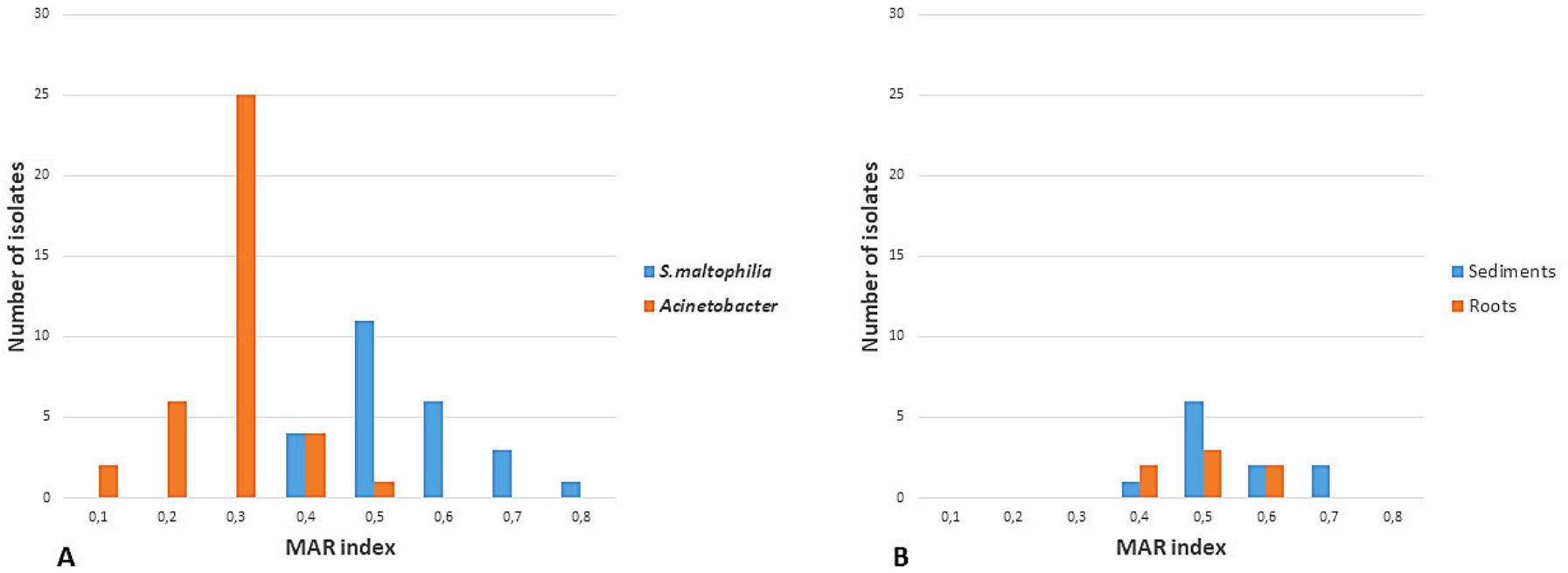


**Figure 5.1 MAR indices of *Acinetobacter* and *S. maltophilia* isolates; Chart A represents indices for *Acinetobacter* and *S. maltophilia* isolates in the water samples and Chart B represents indices for *S. maltophilia* isolates from plant rhizosphere and river sediments**

### 5.3.5 Detection of antibiotic resistance genes/ efflux pump genes

*Sul1* and *sul2* antibiotic resistance genes were found in 24% and 29% of *Acinetobacter* isolates respectively. *Sul3* genes were not detected in any of the isolates from this study. The presence of *sul1* and *sul2* is associated with resistance against trimethoprim-sulphamethoxazole (Sanchez, 2015). In a previous study by Taitt *et al* (2014), 45% and 43% of *Acinetobacter baumannii* isolates were positive for the presence of *sul1*and *sul2* genes. Similar to our findings, no *sul3* genes were detected in any of the isolates from that study. Trimethoprim-sulphamethoxazole is not commonly administered for *A. baumannii* infections but it is imperative that knowledge of their presence is monitored as there is a high potential for the transmission of these resistant genes to other bacteria. This will result in the dissemination of sulphonamide-resistant bacteria in the environment (Manchanda, Sanchaita and Singh 2010).

Beta-Lactamases are enzymes that hydrolyze *β*-lactam antibiotics, and they are the greatest threat to the use of these antibiotics (Bonomo 2017). The $bla_{OXA}$ *23/51* was found in 21% of *Acinetobacter* isolates and 29% of the isolates contained the $bla_{TEM}$ gene. The resistance of isolates towards the beta-lactam antibiotics observed in this study (carbapenems – imipenem, meropenem; ampicillin, ceftazidime, and cefixime**)** could therefore be due to the presence of the $bla_{TEM}$, $bla_{OXA}$ *23/51* as previously reported (Han *et al*. 2017).

*S. maltophilia* isolates were resistant to trimethoprim-sulphamethoxazole but *sul1* genes were present in only 8% of the isolates. Additionally, the $bla_{TEM}$ gene was present in 20% of the *S. maltophilia* isolates. Therefore, other mechanisms could have contributed to the antimicrobial resistance of the remaining isolates, such as multidrug efflux pumps (Sanchez and Martinez, 2018). In this study, the multidrug efflux pumps (s$me_{ABC}$, $sme_{DEF}$) were expressed in all the isolates. It has been reported that overexpression of the $sme_{ABC}$ pump is associated with resistance against aminoglycosides and fluoroquinolones (Herrera-Heredia *et al*. 2017). Overexpression of the $sme_{DEF}$ pumps is associated with resistance against quinolones, tetracyclines, macrolides, chloramphenicol, and trimethoprim-sulphamethoxazole (Chang *et al*. 2015). Since $sme_{DEF}$ can extrude a variety of antibiotics, selection with such antimicrobials, including quinolones, might select for *S. maltophilia* trimethoprim-sulphamethoxazole resistance (Sánchez 2015).

### 5.3.6 Virulence gene detection in *Acinetobacter*

Significant VG of the *A. baumannii* strains of human clinical infections are colicin V production (*cvaC*), curli fibers (*csg*), siderophores like aerobactin (*iutA*), cytotoxic necrotizing factor (*cnf*), overt toxins *espA* (K1 capsular polysaccharide), *ompA* (outer membrane protein A] (Tayabali 2012; Darvishi 2016). In this study, no VG was found in *Acinetobacter* isolates. Virulence of environmental isolates of *Acinetobacter* spp. has been reported based on the assessment of verotoxin and haemolysin production, bacterial serum resistance assay, hydrophobicity, and gelatinase tests (Bagheri Josheghani *et al.* 2015). So far, the presence of VG has not been reported in environmental isolates, however, they have been found in clinical isolates of *Acinetobacter* spp. (Daryanavard and Safaei, 2015; Momtaz, Seifati and Tavakol, 2015; Mohajeri *et al*. 2016).

### 5.3.7 Risks for community-acquired infections

*S. maltophilia* and *Acinetobacter* spp. (especially *A. baumannii*) are well known as nosocomial pathogens (Lowman *et al*. 2008; Bergogne-Bérézin, and Towner, 1996). However, the occurrence of *S. maltophilia* and *Acinetobacter* in environmental samples, such as water, river sediments, and plant rhizospheres as reported in this study, raises questions on their potential in causing community-acquired infections (Cillóniz, Dominedo and Torres 2019; Ferrer *et al*. 2018; Eveillard *et al*. 2013). There is an increasing amount of evidence showing that *S. maltophilia* and *Acinetobacter* can no longer be considered exclusively in nosocomial infections, due to the possibility of causing profound clinical diseases in the absence of risk factors associated with nosocomial infections (Lowman *et al*. 2008). In South Africa, information on community-acquired infections with these two bacteria is scarce. However, a study by McKay and Bamford (2015) reported 39.2% and 54.3% prevalence of community-acquired infections with *Enterobacteriaceae* and Gram-positive organisms, respectively. This follows the first report of community-acquired infection with *A. baumannii* in South Africa (Lowman *et al*. 2008). This indicates that potentially community-acquired infections may be predominant in South Africa however the lack of information could be due to the lack of studies conducted in this area.

It was observed in this study that people within the informal settlements close to the river use the water for several activities. This creates an avenue for exposure to these bacteria and the

possibility of infections thereof. Therefore, the risks associated with community-acquired infections within this scenario need to be addressed. The risk assessment was done qualitatively considering two steps: exposure route and likelihood of infection after exposure.

#### 5.3.7.1 Exposure routes

Exposure to pathogens in water could either be direct or indirect, however in this study we will focus on the direct exposure due to the mode of transmission of the organisms.

A. ***Direct exposure*:** Events such as swimming/bathing in the river or fishing could result in direct exposure to the pathogens. Within the home, direct exposure could be from the use of the river water for different domestic or personal purposes that could lead to direct skin contact or inhalation of aerosols or droplets generated during these activities. It was observed during the study that bathing/swimming in the river and fishing are activities mainly done by young male adults and therefore this part of the population may be at the greatest risks. Use of the water for laundry, cleaning, and other domestic purposes within the home puts women and children at the greatest risks since most of these activities are performed by them.

B. ***Indirect exposure:*** Indirect exposure may occur when infected individuals sneeze or cough, this will generate aerosols. Healthy individuals are therefore at risk of infection when they inhale these droplets, or through direct deposition of droplets from the aerosols in their eyes, nose, or mouth. Generated droplets could travel a distance and land on surfaces or objects and lead to exposure of healthy individuals when they touch these contaminated surfaces with their hands. Every individual within the home setting is at risk of indirect exposure to these pathogens if any member of the household is infected. Therefore, unlike the direct exposure routes, this may be a major route of infection provided there is an infected individual within the household.

#### 5.3.7.2 Likelihood of infection

Exposure to the pathogens in the water, either directly or indirectly will not always lead to an infection. The likelihood of infection is dependent on several factors. These include, but are not limited to:

A. ***Presence at the portal of entry*****:** Community-acquired infections with these pathogens are dependent on burns or wounds where affected skin areas serve as portals of entry for the pathogen leading to infections. The main risk factors for community-acquired infections with these pathogens are a history of trauma, which may provide the portal of entry and infection. For instance, a study in Singapore established that *A. baumannii* colonized burns within 2 days (Eveillard *et al*. 2013). The first case of community-acquired *A. baumannii* infection in South Africa was reported in a patient with a history of trauma from accidents with wounds (Lowman *et al*. 2008). Therefore, the likelihood of infections depends largely on the wounds or burns. The informal settlement is occupied predominantly by unemployed people, who rely on menial labour-intensive jobs to survive. These kinds of jobs make them prone to injuries which may create portals of entry for the pathogens. Secondly, the most exposed part of the population is young male adults who are the most prone to these injuries due to their engagement in physically intensive activities.

B. ***Immune status of exposed populations:*** Like in every infection, the likelihood is dependent largely on the host responses to the pathogen. Community-acquired infection with these pathogens has been implicated in patients with compromised immune systems. South Africa has over 20% of the adult population living with HIV (UNAIDS, 2019) therefore the possibility of immunocompromised individuals being exposed to these pathogens is high. However, the challenge associated with immunocompromised individuals and infections is not peculiar to only infections with *S. maltophilia* and *Acinetobacter*.

## 5.4 Conclusion

This study has shown a high prevalence of *Acinetobacter* sp. and *S. maltophilia* in the environment. Assessment of water from the different sampling points did not show a difference in the impact of the hospital, WWTP, informal settlement and veterinary clinic on concentrations of bacteria. However, analysis of the MAR index from isolates taken from the different points shows a higher selective pressure for antibiotic resistance from the hospital compared to the rest of the sampling points.

The isolates of *Acinetobacter* spp. and *S. maltophilia* displayed MDR against antibiotics commonly used for their treatment. These isolates were more susceptible to fluoroquinolones, levofloxacin and ofloxacin, which may be efficient treatment options in the event of cases or an outbreak. The resistance could have been due to the presence of antibiotic resistance genes in the isolates, as we found, the efflux pumps which have been reported to confer resistance against the antibiotics. The findings in this study are significant due to the potential of ARG harboured by *Acinetobacter* and *S. maltophilia* to be transferred to other bacteria via horizontal gene transfer. Additionally, the bacteria with ARG can flourish and be widely distributed in the environment, thus posing a risk to human health. Despite the detection of *Acinetobacter* with antibiotic-resistant properties, no VG was detected.

Additional findings made in this study show that river water quality monitoring should include analysis of the river sediments and plant rhizosphere. We found that *S. maltophilia* was more prevalent in these matrices than in the water samples.

The risk of infections with these MDR *Acinetobacter* and *S. maltophilia* outside the hospital setting is high. Conditions within the study area are prime for these community-acquired infections. Therefore, suggest public awareness is recommended as a major tool to limit exposure to the river. For instance, the public could be warned to reduce swimming, fishing and other activities in sections of the river with high contamination, such as downstream of the hospital.

# Chapter 6: Application of Flow Cytometry and droplet digital PCR as Rapid Methods for Wastewater Analysis

## 6.1 Introduction

Conventional methods used for the assessment of microbial water quality are mainly based on selective culture media and standard biochemical-based methods (Farhat *et al*. 2020). Although culture-based methods are simple and low cost, they have low sensitivity and are laborious and time-consuming (Ramírez-Castillo *et al.* 2015; Porcellato, Narvhus and Skeie, 2016). In addition, certain bacteria, although viable, may exist as VBNC, leading to false negatives. These may tentatively still occur in infective quantities in the environment (Cenciarini-Borde, Courtois and La Scola, 2009).

The challenges experienced with conventional culture-based methods call for timely and advanced molecular-based methods such as FISH, PCR, and FCM, for the detection and quantification of potential pathogens. The FISH technique is built on cell-based quantification of microorganisms at different taxonomic levels, depending on the degree of conservation of the target sequence probe (Amann, Ludwig and Schleifer 1995). On the other hand, FCM allows for a high throughput quantification and the phenotypic separation of cell populations based on differences in the surface characteristics of single cells (Müller and Nebe-von-Caron, 2010). This technique has been in use for over two decades and has been proven efficient in water quality monitoring (Safford and Bischel 2019). Similarly, staining with DNA intercalating fluorochromes such as propidium iodide (PI) and SYTO 9, which are part of the BacLight kit can distinguish between live and dead cells using membrane integrity as an indication of cell viability (Berney *et al*. 2007; Stiefel *et al*. 2015). The BacLight kit uses a dual staining technique whereby both live and dead cells are permeable to SYTO 9 and green fluorescence is emitted. PI, on the other hand, permeates damaged or dead cells, and red fluorescence is emitted (Berney *et al*. 2007; Freire *et al*. 2015). The BacLight kit is also able to determine the concentration of live and dead bacterial cells in a sample. However, these dyes cannot taxonomically identify bacterial cells in a mixed sample as they are not specific (Amann *et al*. 1990). To overcome these limitations, researchers have also tried alternate methods such as Flow-FISH which is a combination of the specific binding technique of FISH and the high throughput quantification approach of FCM which allows the identification and quantification

of specific bacteria. Flow-FISH has been used successfully for the analysis of microbial community structures in environmental samples (Rufer *et al.* 1998; Friedrich and Lenke 2006; Nettmann *et al.* 2013).

ddPCR is an advanced PCR-based method that has recently been gaining attention as it can provide accurate quantification without the need for a standard curve (Hindson *et al.* 2011; Porcellato, Narvhus and Skeie 2016). The technique is based on microfluidics technology that allows for the generation of multiple reaction partitions that occur as individual reactions. It follows the Poisson distribution through which it is possible to determine the concentration of selected target bacteria as the number of copies per microliter in the reaction (Huggett *et al*. 2013; Morisset *et al*. 2013). Currently, this technique has been applied for the detection and quantification of pathogens from different matrices such as food (Bian *et al.* 2015), soil (Dong *et al.* 2014), bovine faeces (Verhaegen *et al.* 2016), and water (Cao, Raith and Griffith 2015; Te, Chen and Gin 2015). The most recent application of ddPCR was to detect SARS-CoV-2 in clinical (Dong *et al.* 2020; Suo *et al.* 2020) and environmental samples such as wastewater (Pillay *et al*. 2021).

This chapter, therefore, focuses on the use and comparison of advanced techniques such as FCM for Flow-FISH, bacterial viability studies, ddPCR, and total counts, for the detection and quantification of the selected bacterial pathogens from wastewater influent and effluent.

## 6.2 Methodology

### 6.2.1 Sample collection

Composite samples of influent (untreated) (*n*=3) and the final effluent (treated) (*n*=3) were collected from the NWWTP which treats domestic, industrial, and hospital effluents in the city of Durban. For the composite sample of 3 litres, 750 mL aliquots of wastewater influent and effluent were collected every 15 min over sixty min. Composite samples were taken to ensure that the wastewater sample was representative and integrated. The samples were decanted into 5 L bottles, separately, for both influent and effluent and transported to the laboratory on ice. The samples were mixed well and divided into three separate one-litre sterile bottles and stored at 4°C for further analysis.

### 6.2.2 Plate count enumeration

The total plate counts were performed using Nutrient agar, and for each of the target bacteria, selective media was used, and this is described in detail in **Chapter 3: Section 3.4.1.**

### 6.2.3 Control cultures for Flow-FISH and ddPCR

Pure cultures of *Acinetobacter baumannii* (ATCC 19606), *Pseudomonas aeruginosa* (ATCC 27853), *Aeromonas hydrophila* (ATCC 7966), and *Stenotrophomonas maltophilia* (13637) were cultured under defined cultivation conditions as control samples. These cultures were then grown in Nutrient Broth (Oxoid, England) at 37°C for 24 h to approximately $10^6$ cells per mL and used for FCM viability studies, Flow-FISH and ddPCR method development.

### 6.2.4 Quantification and detection of selected target bacteria using droplet digital PCR

#### 6.2.4.1 DNA extraction of pure cultures of selected target bacteria

DNA extraction methods have been described previously in **Chapter 3: Section 3.8.1**.

#### 6.2.4.2 DNA extraction from wastewater influent and effluent samples

The wastewater influent (500 mL) and effluent (1000 mL) samples were centrifuged (3000 rpm for 3 min) at 25°C and the pellet was used for the extraction of DNA. Briefly, the pellet was placed in a PowerBead tube (provided in the kit), and bead-beating was performed at medium

speed for 3 min (Omni Bead Beater, USA). The samples were then centrifuged at 10,000 x *g* for 30s, and the supernatant was transferred to a clean 2 mL collection tube. Thereafter, the DNA was extracted following the DNeasy PowerSoil kit (Qiagen, Germany) manufacturer's instructions. The final elution of DNA was done using Tris-EDTA buffer. The DNA concentration and purity were measured with a nanophotometer (Implen, Germany). The DNA extractions were done in triplicate. Each tube with purified DNA was sealed with Parafilm (Bemis Company, USA) and stored at -20°C for further use.

#### 6.2.4.3 Limit of detection for droplet digital PCR assay

Serial dilutions of the extracted DNA of each pure culture (*Acinetobacter* spp., *Aeromonas* spp., *Pseudomonas* spp., and *S. maltophilia*) were made using nuclease-free water. The dilutions ranged from 2.5 ng/µL to 1.25 fg/µL. The dilutions of DNA were used as the template for ddPCR reactions and were performed as explained in **Section 6.2.5.4**. The measured copies were generated using QuantaSoft™ (Bio-Rad) software.

#### 6.2.4.4 Droplet digital PCR analysis of wastewater samples

The ddPCR analysis was performed in triplicate using the QX200 ddPCR system (Bio-Rad, USA). The reaction mixture was prepared in a final volume of 20 µL, which consisted of 10 µL of QX200™ EvaGreen Supermix [no deoxyuridine triphosphate (dUTP)] (Biorad, USA), 2 µM of each primer, 6 µL nuclease-free water (Thermofisher, USA), and 2 µL template DNA (5 ng). Droplets were generated using an automated droplet generator [QX200$^{TM}$ AutoDG ddPCR system (Bio-Rad, USA)]. The plate containing the droplets (40 µL) was carefully removed from the automated droplet generator. The plate was then heat-sealed and loaded into a C100 Touch$^{TM}$ Thermal Cycler (Bio-Rad, USA) for amplification. The thermal cycling conditions for the selected target bacteria (*Acinetobacter* spp., *Pseudomonas* spp., *Aeromonas* spp., and *S. maltophilia*) with the addition of a ramp rate of 2°C/s at every step are presented in Table 4.1. and 5.1. After thermal cycling, the plates were loaded into a QX200$^{TM}$ Droplet Reader (Bio-Rad, USA). The ddPCR analysis included positive template controls (PTCs) and negative template controls (NTCs) in triplicate. NTCs used in the reaction mixes were nuclease-free water. PTCs included gDNA extracted from reference strains listed in **Section 6.2.3**. The QuantaSoft™ Analysis Pro software (version 1.0.596) (Bio-Rad, USA) was used

for the analysis of the data. Thresholds were set manually for each sample using criteria defined for each assay.

### 6.2.5 Application of Flow Cytometry for wastewater analysis

#### 6.2.5.1 Viability assay of reference control cultures using the Baclight kit

Viability was determined by the analysis of membrane integrity using the BacLight kit (Invitrogen, 2004) in combination with FCM. The single colour controls (live cells and ethanol-treated dead cells) that were prepared from pure cultures were used for setting and adjusting the flow cytometer.

Pure cultures of *Pseudomonas aeruginosa* were used to optimize the flow cytometer for the live/dead analysis as this species showed a good correlation between the BacLight kit (Invitrogen, Molecular Probes, USA) and plate counts (Invitrogen, 2004). Bacterial suspensions were prepared as follows: pure cultures of *Pseudomonas aeruginosa* were grown overnight at 37°C in Nutrient Broth (Oxoid, England). The culture was concentrated by centrifugation at 10 000 x *g* for 10 min. The supernatant was removed, and the pellet was resuspended in 2 mL of 0.85% NaCl. The suspension (1 mL) was then added to a tube containing 1 mL of 0.85% NaCl, which served as the control for live bacteria. The control for dead bacteria contained 1 mL of bacterial suspension and 1 mL of 70% ethanol. Both suspensions (live /dead) were incubated at room temperature (25°C) for 60 min and were vortexed every 15 min. Both the live and dead suspensions were then centrifuged and washed in 0.85% NaCl. Each pellet (live and dead) was then resuspended in 1 mL of 0.85% NaCl. The live and dead controls were used for setting the gates for the flow cytometric viability analysis using the BacLight kit (Invitrogen, Molecular Probes, USA).

#### 6.2.5.2 Wastewater sample preparation and staining protocol using the BacLight kit

Five hundred millilitres of the influent and effluent wastewater samples were mixed thoroughly and filtered through 100 µm and 20 µm sterile test sieves (Retsch, Germany) to remove debris. Aliquots (990 µL) of samples were used for the determination of bacterial viability using the BacLight kit (Invitrogen, Molecular Probes, USA). The kit consists of a microsphere suspension and two fluorescent dyes, SYTO 9 and PI that label live and dead bacteria, respectively. The dyes (SYTO 9 and PI) are stored in Dimethyl sulfoxide at a concentration of

3.34 mmolL and 20 mmolL, respectively. The microspheres are suspended in deionised water containing sodium azide, at a concentration of 1 $\times 10^8$ beads per mL. The microsphere suspension was homogenised by gentle vortexing before use.

The staining procedure was as follows; 1 µL of SYTO 9 and 1 µL of PI was added into a microcentrifuge tube. Thereafter 10 µL of the microsphere suspension and 988 µL of the wastewater samples were added into the above mixture. The tubes were thereafter incubated in the dark for 15 min at 25°C to allow for the binding of the dye to the bacteria in the sample. The samples were gently mixed after incubation.

#### 6.2.5.3 Flow cytometric analysis

The flow cytometric analysis was performed on the FACS ARIA III (BD Biosciences, USA). The fluorescence of the probes was excited at a wavelength of 488 nm. The SYTO 9 fluorescence was measured using a 505 nm long-pass filter and a bandpass filter with transmission at 530/30 nm. The PI fluorescence was measured using a 685 nm long-pass filter and bandpass filter with 695/40 nm transmission. The threshold was set to side scatter (SSC) at 200 to minimise the effect of noise. The photomultiplier (PMT) tube voltage was adjusted to ensure that both the bacterial populations and beads were on scale in an SSC vs forward scatter (FSC) plot. A histogram was incorporated in the analysis to observe the bead count consistency. The instrument was calibrated, before use with BD FACSDIVA™ Cytometer Setup and Tracking Research Beads (BD, USA), according to the manufacturer's instructions. The laser output, voltage, and gains of the detectors were adjusted to gate the bacterial populations of interest, with maximum separation from sample debris. The live and dead bacteria were analysed by gating the populations in the red fluorescence (dead) versus green fluorescence (live) in the plots. For the pure culture, the bacterial concentration was kept to a maximum of $10^8$ bacteria mL. To maintain a constant setting for all measurements while reducing the occurrence of coincident detection of bacteria in the high concentration samples, the flow rate was kept constant, and each measurement was 10 000 events. The addition of microsphere beads was used for the calculation of the absolute concentration of the bacteria measured with FCM.

The following formula describes the calculation for the concentration of live bacteria (Invitrogen, 2004).

$$concentration\ of\ live\ bacteria$$

$$= \frac{no.of\ events\ in\ live\ region}{no.\ of\ events\ in\ bead\ region}\ x\ conc.of\ beads \times dilution\ factor$$

Where "*no. of events in the live region*" represents the cells stained by SYTO 9 (which excludes dead cells). In the equation above, "*conc. of beads*" refers to the bead concentration measured in the entire sample volume, "*no. of events in bead region*" refers to the number of microsphere events which is used to measure the volume of sample analysed and the "*dilution factor*" refers to the diluted bacterial sample. Each sample was measured in triplicate.

#### 6.2.5.4 Flow-FISH: Preparation and fixation protocol of reference control cultures and wastewater samples

Control cultures were prepared as described in **Section 6.2.3**. The influent (500 mL) and effluent (1000 mL) samples were filtered as described above in 6.2.4.2. The control cultures (5 mL) and the filtered wastewater samples were pelleted at 3000 rpm for 10 min in a centrifuge. Fixation was carried out on 5 mL of the pellet (control cultures and wastewater sample), according to a protocol reported by (Amann, Ludwig and Schleifer 1995). Samples were fixed in 4% formaldehyde solution (diluted in 1× PBS pH 7.4) for 3 h at 4°C. After fixation, the samples were centrifuged at 8,000 × g for 20 min at 25°C. The supernatant was discarded, and the pellet was washed twice in 1× PBS using the centrifugation conditions as before. After washing, the pellet was resuspended in 5 mL 1× PBS, mixed with 5 mL of 96% ethanol and stored at −20°C until further use.

##### 6.2.5.4.1 Flow-FISH: FCM analysis

FISH was carried out using 16S rRNA targeted probes (See Table 6.1) that are specific for the different groups of bacteria targeted in this study (*S. maltophilia*, *Acinetobacter* spp., *Pseudomonas* spp., and *Aeromonas* spp.). All FISH probes were labelled fluorescently with 6-*FAM* (Fluorescein) and were synthesized by Integrated DNA Technologies (Cape Town, South Africa). The positive controls were fixed reference cultures described in **Section 6.2.3**. Negative controls (without the addition of probes) were used to determine the effect of autofluorescence. The modified protocol by Nettmann *et al.* (2013), was used for the preparation of both wastewater samples and control cultures as follows: 250 µL of the fixed

sample was centrifuged at 8,000 × *g* for 20 min at 25°C and the supernatant was discarded. The pellet was re-suspended in preheated (46°C) hybridization buffer [0.9 M NaCl, 20 mM Tris/HCl (pH 7.2), 0.1% SDS, 35% formamide]. An aliquot of 21 µL of the FISH probe (50 ng/µL) was added to the pelleted sample and was incubated at 46°C for 2 h. During incubation, the samples were repeatedly inverted in a hybridisation oven to ensure the even distribution of probes. After incubation, the samples were centrifuged at 8,000 × *g* for 20 min to pellet the cells. The pellet was washed twice with 500 µL 1× PBS (Oxoid, England). Thereafter the pellet was resuspended in 500 µL of hybridisation buffer and incubated for 20 min in the dark. This was followed by centrifugation for pellet formation. The pellet was resuspended in 500 µL of wash buffer and incubated at 25°C for 20 min. The purpose of this step was to remove the hybridisation buffer and non-bound probes from the samples. The samples were then centrifuged, and 1 mL of cold PBS was added to each pellet. This was vortexed for 30 s to homogenise the pellet. The pelleted samples were further diluted in 1× PBS for subsequent flow cytometric analysis.

**Table 6.1 Probes used in this study**

| Target Bacteria | Probe Sequence (5'-3') | Target site | Reference |
|---|---|---|---|
| ***Pseudomonas*** | GATCCGGACTACGATCGGTTT | 16S rRNA | (Gunasekera *et al.* 2003) |
| ***Aeromonas*** | ACCTGGGCATATCCAATC | 16S rRNA | (Kämpfer et al. 1996) |
| ***Acinetobacter*** | GCGCC ACTAA AGCCT CAAAG GCC | 16S rRNA | (Kenzaka *et al.* 1998) |
| ***S. maltophilia*** | GTCGTCCAGTATCCACTGC | 16S rRNA | (Hogardt *et al.* 2000) |

### 6.2.6 Statistical analysis

All data are expressed as mean ± standard deviation (SD) from the triplicate analysis of plate counts, FCM counts, and ddPCR analysis. Statistical analyses of data were performed using the Student's paired t-test. A significant difference is defined as $p$ value$\leq$0.05.

## 6.3 Results and discussion

This part of the study explored the applicability of FCM (viability/total counts, Flow-FISH) and ddPCR in relation to the plate count method for the detection and quantification of selected bacteria from wastewater samples. Although these are established methods, there are limited studies that have used FCM and ddPCR to detect and quantify the selected target bacteria under investigation from wastewater.

### 6.3.1 Enumeration of selected target bacteria from wastewater influent and effluent using the conventional plate count method

Results obtained for the total plate count for wastewater samples analysed are depicted in Table 6.2 and Figure 6.1. The total plate counts for the wastewater influent and effluent samples averaged at 6.46±0.02 and 4.6±0.7 ($\log_{10}$ CFU/mL); $p\leq0.05$, respectively. Among the four targeted bacteria, *Aeromonas* (influent: 5.31±0.17, effluent: 3.7±0.2; $\log_{10}$ CFU/mL; $p\leq0.05$) was found in the highest concentration whilst *S. maltophilia* was found in the lowest concentration (influent: 3.82±0.3; effluent: 3.16±0.2 ($\log_{10}$ CFU/mL); $p\leq0.05$). A significant difference ($p\leq0.05$) in the bacterial count was observed between the influent and effluent samples for all targeted bacteria except for *Acinetobacter*.

Traditional enumeration methods, such as the plate count method, have been reported as an acceptable method to measure water quality (Allen, Edberg and Reasoner 2004, Cheswick *et al*. 2019). The method has also been approved by regulatory bodies (ISO, 2006); however, it has many drawbacks. Species or strains with similar colony morphology are not easily distinguished from each other (Herbel *et al*. 2013). In addition, the method is quite laborious and time-consuming (Deshmukh, 2016). It was also reported that the bacteria that can enter the VBNC state under environmental stress, such as during chlorination, will go undetected using

the plate count method (Chen *et al*. 2018). This may result in an underestimation of the bacterial count, which can increase the risk of waterborne illness.

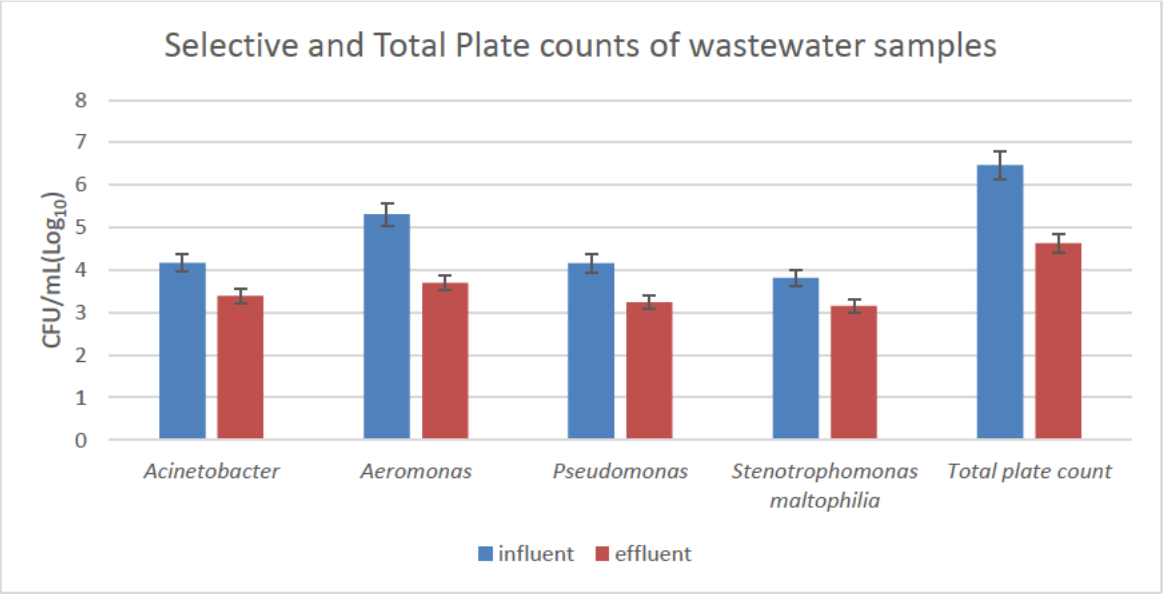


**Figure 6. 1 The enumeration of selected target bacteria and the total bacteria from wastewater influent and effluent using the plate count method (*n*=3)**

### 6.3.2 Droplet Digital PCR

#### 6.3.2.1 Application of droplet digital PCR to detect *Acinetobacter* spp., *Aeromonas* spp., *Pseudomonas* spp., and *S. maltophilia* from influent and effluent samples

The ddPCR method was used for the absolute quantification of the selected target bacteria in the wastewater samples using species-specific primer sets (Table 4.1; Table 5.1.). The limit of detection (LOD) was performed for each of the target bacteria (*Acinetobacter*, *Aeromonas*, *Pseudomonas*, and *S. maltophilia*) using gDNA from pure cultures to evaluate the sensitivity of the assay. The LOD was determined from the lowest concentration amplified from the triplicate analysis of serial dilutions of the gDNA from the pure cultures. The LOD (GC/µL) determined for each of the different bacterial targets were as follows [*S. maltophilia* (0.15), *Acinetobacter* (0.6), *Aeromonas* (1.4) and *Pseudomonas* (0.65)]. Negative controls were analysed for each of the LOD assays in ddPCR, and this was used to manually set a threshold.

Using ddPCR analysis (Table 6.2. and Figure 6.2.), it was found that the bacteria in the highest concentration ($\log_{10}$ GC/mL) was *Pseudomonas* (influent:7.19±0.24 GC/mL; effluent: 6.48±0.2 GC/mL). *S. maltophilia* was found in the lowest concentration in both influent (5.40±0.90 $\log_{10}$ GC/mL) and effluent (4.53±0.57 $\log_{10}$ GC/mL). This trend was similar to the plate count method where *S. maltophilia* was found in the lowest concentration from both influent and effluent samples. The ddPCR results showed that *S. maltophilia* and *Acinetobacter* were detected in a lower concentration in influent samples in comparison with FCM. This was not expected and could be because of sample processing biases (filtering, concentration, DNA extraction) whereby the final yield and quality of DNA were impacted and led to inaccuracies (Martinez-Hernandes *et al*. 2019). In comparison to the selective plate counts for each targeted bacterium, the ddPCR results indicated higher concentrations of all these bacteria both in the influent and effluent samples as expected. This was demonstrated in studies by Ahn *et al*. 2014 where *Burkholderia cepacia* was detected in lower concentrations in nuclease-free water using the plate count method in comparison to ddPCR.

Both these methods work on different principles, whereby plate counts measure the viable bacteria, whereas the ddPCR can detect both live and dead bacterial cells as this method relies mainly on amplifying the DNA in the samples. The DNA-based ddPCR technique cannot differentiate between live and dead cells, which is regarded as one of the limitations of this

technique when applying it to environmental samples (Gobert *et al*. 2018; Santander, Meredith and Aćimović, 2019). The use of propidium monoazide pre-treatment to prevent the amplification of DNA associated with dead cells has been proposed as an alternative technique to improve the accuracy of ddPCR in detecting DNA from viable cells (Kiefer, 2020). To the best of our knowledge so far, there are no studies that used ddPCR to detect and quantify the selected target bacteria in wastewater samples. Most of the available data is from clinical samples (Morella *et al*. 2018; Ziegler *et al*. 2019; Wouters *et al*. 2020).

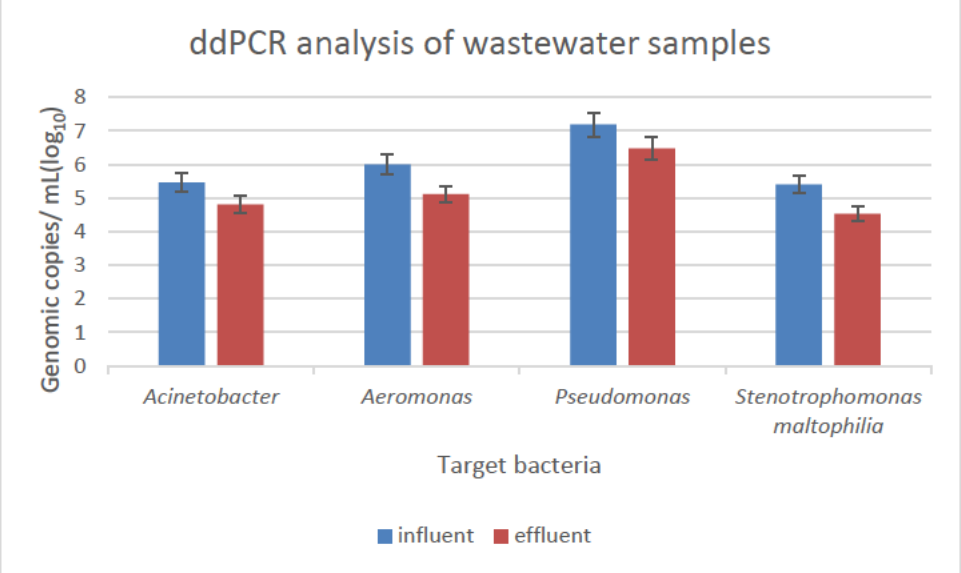


**Figure 6.2 Genomic copies of selected target bacteria in wastewater influent and effluent samples (*n*=3)**

### 6.3.4 Flow Cytometry

FCM with the use of the BacLight kit was used to determine the total (viable/ non-viable) count of wastewater influent and effluent. Each of the selected target bacteria present in the wastewater was enumerated using the Flow-FISH technique. FCM uses laser technology and the principle of fluorescence to identify, quantify and isolate cells from a heterogenous mixture such as wastewater. For the Flow-FISH assay, existing FISH methodology and probes specific for the selected target bacteria were used (Table 6.1.) for detection and quantification.

#### 6.3.4.1 Enumeration of total and viable bacteria in samples using the BacLight kit

##### 6.3.4.1.1 Optimization of the viability analysis using pure cultures

Pure cultures of the selected target bacteria were used for adjusting the flow cytometer to ensure the PMT voltages were set. The viable bacterial cells demonstrated strong green fluorescence (SYTO 9) and no red fluorescence (PI), while a completely permeabilized population (heat-treated) showed a weak green fluorescence and a strong red fluorescence signal (Figure 6.3). The PI and SYTO 9 dye used in this study provided good resolution between the live and dead cells. These cell populations were defined by the drawing of rectilinear regions around the live and dead cells. This gated region was used as a template for the viability analysis and total counts of the wastewater samples that followed. Previous studies have demonstrated that PI and SYTO 9 staining allowed the differentiation of live and dead cells (Stiefel *et al*. 2015; Ou *et al*. 2017).

##### 6.3.4.1.2 The application of the BacLight kit to enumerate wastewater samples

The Baclight kit demonstrated that the wastewater samples had an average total bacterial count (live/dead) ($log_{10}$ bacteria/mL) that ranged from 8.49±0.03 - 7.98±0.04 (influent) and 7.38±0.02 - 7.13±0.02 (effluent). Of this total count of bacteria, the viable population range ($log_{10}$ bacteria/mL) was 8.00±0.02 - 7.72±0.05 (influent) and 6.84±0.11 - 6.60±0.04 (effluent). A statistically significant difference ($p \leq 0.05$) was observed between the total count and viable count of the wastewater samples (Table 6.2.). In this investigation, it was made clear that there was a statistically significant difference between the total viable count using FCM and the BacLight kit in comparison to the culture-based total plate count ($log_{10}$ CFU/mL). This is expected, as the plate count only detects a fraction of the viable and culturable bacteria, whilst with the BacLight kit and FCM, the VBNC bacteria will also be detected. These results suggest that flow cytometric total counts can be used as a culture-independent strategy for monitoring bacterial concentration and viability. It also allows for the quantitative viability analysis of bacteria that are unculturable or bacteria that have entered a VBNC state. In a similar study on wastewater influent and effluent, Manti *et al.* (2008) reported that total count data obtained by FCM was significantly higher than those measured with traditional plate counts. Another study by Ou *et al.* (2017) showed that FCM measured 0.273 $log_{10}$ bacteria/mL more bacteria than plate counts. Similar trends were also reported in other studies (Bensch *et al*. 2014; Buzatu *et al*. 2014).

In a study by Li *et al.* (2007) where hospital wastewater was investigated, it was found that counts using FCM were up to 67 times higher than the culture-based methods. This demonstrated that the culture-based method commonly used for water quality monitoring underestimates the actual counts and is therefore inadequate. FCM has mainly focused on activated sludge samples in monitoring WWTPs activated sludge (Foladori, Tamburini and Bruni, 2010; Abzazou *et al*. 2015). Similarly, Collado *et al.* (2017) used FCM to investigate the viable portion of activated sludge and found that viable cells were 47% of the total population of cells. When monitoring bacterial concentrations, it is important to determine the viability of the bacteria and their abundance as both are critical in assessing water quality (Pianetti *et al*. 2005). The BacLight kit is a rapid method since we were able to get results for both the viable and total counts in a single step. It can detect the VBNC bacteria that adds to the reliability of the assay (Boulos *et al*. 1999). However, there are limitations to the use of the BacLight kit, one of which is the ability to differentiate between viable and dead cells, as the assay is based entirely on membrane integrity (Fleischmann *et al*. 2021).

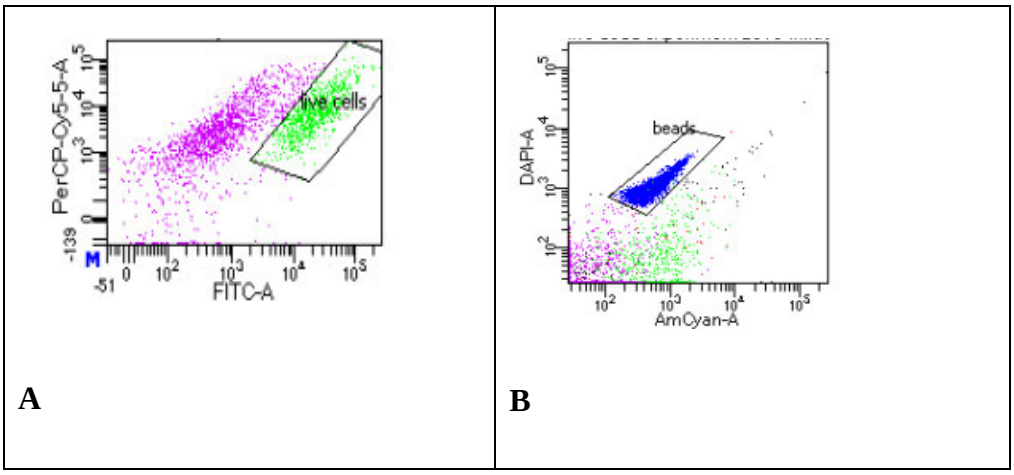


**Figure 6.3A - Dot plot of gating viable cells which are in the gate labelled live cells (PI negative). The cells adjacent to these gated are non-viable and are purple. B – Dot plot of gating microsphere beads that are used to determine the sample concentration.**

#### 6.3.4.1.3. Detection and quantification of selected target bacteria using Flow-FISH

Flow-FISH has not been used earlier to detect and quantify selected target bacteria that are part of this study to the best of our knowledge. Previously designed FISH probes, for 16S rRNA-targeted detection of *Acinetobacter* spp., *Pseudomonas* spp., *Aeromonas* spp., and *S. maltophilia* (Table 6.1.), were evaluated for applicability of use by performing *in situ* hybridization experiments with pure cultures of *Acinetobacter baumannii, Aeromonas hydrophila*, *Pseudomonas aeruginosa*, and *Stenotrophomonas maltophilia*. Examination of the fluorescently stained cultures using fluorescent microscopy showed successful hybridization of the probes to the selected target bacteria confirming probe specificity.

All the target organisms were detected in the influent and effluent samples, with the average concentrations being higher in the influent (Table 6.2 and Figure 6.4). S. *maltophilia* was detected in the highest concentration, in both influent and effluent ($log_{10}$ bacteria /mL) of 5.92± 0.02 and 4.31±0.15; ($p \leq 0.05$), respectively in comparison to plate counts and ddPCR. A previous report by Hoefel *et al*. (2005), demonstrated that *S. maltophilia* can maintain membrane integrity, but it develops recalcitrance to plate cultures, and this bacterium is known to enter a VBNC state. Similarly, Flow-FISH detected *Pseudomonas* species in lower concentrations in both influent and effluent wastewater ($log_{10}$ bacteria /mL) [influent (5.41±0.07), effluent (3.43±0.02) $p \leq 0.05$] as opposed to the ddPCR results, which displayed *Pseudomonas* as the dominant bacteria in these samples. A similar trend was also seen for *Aeromonas* spp. but there was no significant difference observed between the influent and effluent concentrations (Table 6.2.). In general, the

results for Flow-FISH were found to be higher in concentration (between 0.2-2.1 log) for each of the selected target bacteria than the plate counts (Table 6.2.), but lower than the concentration obtained using ddPCR, in most cases. These results are in accordance with other studies where traditional plate counts produced a lower count than FCM (Mezzanotte *et al*. 2004; Manti *et al*. 2008). Flow-FISH can detect bacteria that are in the VBNC state while traditional plate counts may not be able to detect bacteria in this state, which poses a risk as the bacteria are capable of retaining virulent properties (Baffone *et al*. 2003; Fakruddin *et al*. 2013).

A combination of FCM and phylogenetic probes have been successfully applied to describe bacterial communities in environmental, clinical, and artificial samples, such as human faeces (Rigottier-Gois *et al*. 2003), ballast water (Joachimsthal *et al*. 2004, Khandeparker *et al*. 2018), and activated sludge (Brown *et al*. 2019). In all studies above, Flow-FISH was found to be a reliable method that gave consistent and complementary results compared to other established methods.

Despite limitations of the use of FCM which is discussed later in this chapter, combining the rapidity and multi-parametric accuracy of FCM and the phylogenetic specificity of oligonucleotide probes using the FISH technique has become an essential tool in monitoring aquatic matrices when targeting specific bacteria (Manti *et al*. 2011).

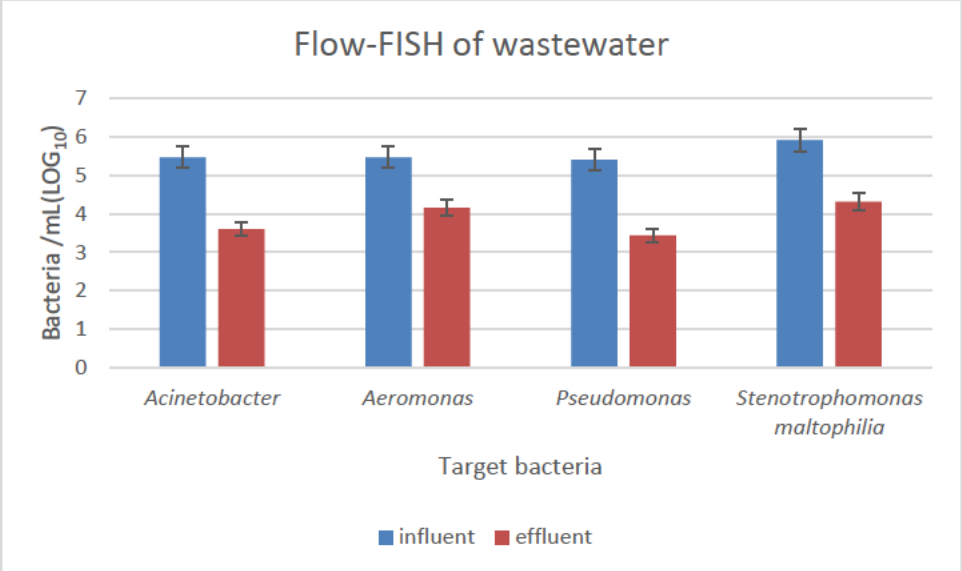


**Figure 6.4 Flow-FISH quantification of selected target bacteria in wastewater (*n*=3)**

**Table 6.2 Average count of selected target bacteria in wastewater influent and effluent using different enumeration techniques (*n*=3)**

| **Method of analysis** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Plate count** | | | **Flow-FISH** | | | **ddPCR** | | |
| **Target bacteria** | **Average Count($log_{10}$)** | | | | | | | | |
| | **CFU/mL** | | ***p* value** | **Bacteria/mL** | | ***p* value** | **GC/mL** | | *p* value |
| | **Influent** | **Effluent** | | **Influent** | **Effluent** | | **Influent** | **Effluent** | |
| ***Acinetobacter* spp.** | 4.17±04 | 3.39±0.01 | $p>0.05$ | 5.47±0.03 | 3.60 ±0.6 | $p\leq0.05$ | 5.47±0.5 | 4.81±0.37 | $p>0.05$ |
| ***Aeromonas* spp.** | 5.31±0.17 | 3.70±0.20 | $p\leq0.05$ | 5.47±0.36 | 4.16±0.1 | $p>0.05$ | 6.00±0.27 | 5.11±0.63 | $p>0.05$ |
| ***Pseudomonas* spp.** | 4.16±0.18 | 3.24±0.02 | $p\leq0.05$ | 5.41±0.07 | 3.43±0.2 | $p\leq0.05$ | 7.19±0.24 | 6.48±0.20 | $p>0.05$ |
| ***Stenotrophomonas maltophilia*** | 3.81 ± 0.35 | 3.16±0.17 | $p\leq0.05$ | 5.92±0.02 | 4.31±0.15 | $p\leq0.05$ | 5.4±0.90 | 4.53±0.57 | $p>0.05$ |
| **Total plate count** | 6.46± 0.02 | 4.63 ±0.7 | $p\leq0.05$ | Not applicable | | | Not applicable | | |

Overall, the different techniques used in this study have both advantages and disadvantages. One of the major advantages of traditional heterotrophic plate counts is that a positive result is an indicator of the viability of the bacteria that form colonies (van Nevel *et al*. 2017). This is a simple and low-cost method and does not require expensive equipment or skills to perform the analysis (Douterelo *et al*. 2014). Although plating or culture-based methods are referred to as the gold standard, they are laborious and time-consuming, and VBNCs are not recovered using this method (Deshmukh, 2016), which can lead to underestimating the actual pathogen concentration in water quality monitoring programs.

FCM, on the other hand, is capable of detecting VBNC bacteria. FCM allows the analysis of thousands of bacterial events in a few seconds, while traditional methods may take a few days. This method is rapid, there is the ease of sample preparation, and it has a high counting efficiency (Brown *et al*. 2015). FCM, in combination with fluorescence-based approaches such as FISH, allows for the differentiation of specific bacteria in a sample (Nettman *et al*. 2013).

The main limitations of FCM are the need for well-trained operators, the high cost of equipment and consumables (fluorochromes, control organisms) to run assays (Zhang and Liang 2014; Betters *et al*. 2015). Optimal sample preparation is essential and dependent on the type of sample, the target organisms, and the instrument (Safford and Bischel, 2019). Fluorochrome selection is another limitation as various factors such as binding specificity/ efficiency, excitation/ emission spectra, and the overlapping of spectra influence the staining protocol need to be considered (Shapiro *et al*. 2003). The interpretation of viability data is also limiting as there is no universally accepted method for assessing viability. Viability analysis using FCM is normally assessed with the combination of a cell-permeable nucleic acid stain (SYTO/SYBR stains) and a cell impermeable stain (PI), in certain instances, the above-mentioned stains are not able to distinguish between populations that have intact or compromised membranes and sometimes intermediate states are detected (Berney *et al*. 2007; Kaur *et al*. 2013). Although these limitations are present, FCM analysis can be strengthened when coupled with other methods such as traditional heterotrophic plate count (Ou *et al*. 2017), epifluorescence microscopy (Brown *et al*. 2019), molecular techniques such as ddPCR (Hansen *et al*. 2020), ATP determination (Vignola e*t al*. 2018), and assimilable organic carbon measurement (Tang *et al*. 2018).

While ddPCR has emerged as a reliable tool for detecting pathogens from samples with a complex mix of inhibitors and low concentrations of target DNA such as wastewater (Singh *et al*. 2017). However, there are still limitations that prevent the universal use of this method. These include sample processing which could impact the yield of DNA and lead to incorrect quantification of target organisms. The specificity of the primer or probe used is also important to ensure we have specific data to detect the target organism (Martinez-Hernandez *et al*. 2019).

The detection of genetic material (DNA) associated with the presence of pathogens using ddPCR is another limitation whereby direct inferences cannot be made to link the presence of the pathogen with its viability status. Despite the limitations, this method is sensitive, specific, and rapid and hence makes it an important tool in the detection of waterborne opportunistic pathogens.

## 6.4 Conclusion

Rapid, advanced methods for detecting potential waterborne opportunistic pathogens to eliminate the risk posed by them in water matrices are in need. Most studies use conventional methods such as the traditional plate count, mainly due to its economic feasibility and ease of use. This study focused on the applicability of three established methods that is total plate counts, FCM, and ddPCR for the detection and quantification of the targeted bacteria that are part of this study. Among the methods used, it was found that the plate count method measured the lowest bacterial counts while ddPCR measured the highest concentration of bacteria. ddPCR has additional benefits to plate counts in that it has high precision, is rapid, and is strain-specific. In this study, ddPCR measured both live and dead bacteria to give an absolute count.

However, it has also been demonstrated in this study that FCM will be of more benefit in water analysis as it can be used to measure bacterial concentration and when combined with FISH, it can distinguish between different types of bacteria in samples. FCM total counts are more suitable for process monitoring in comparison to culture-based methods which are sporadic. These different methods used in tandem in the water industry will provide diverse information which together will give a complete overview of the composition of samples.

# CHAPTER 7: Summary and conclusions

## 7.1 Summary

The rapid emergence and widespread incidence of MDR in bacteria is a global occurrence, and this has resulted in compromising the efficacy of antibiotics. This has led to antibiotic resistance becoming a public health concern.

The focus of this study was on antibiotic resistance in *Aeromonas* spp., *Stenotrophomonas maltophilia,* and two of the genera which have species deemed to be priority 1: critical pathogens (*Pseudomonas* spp. and *Acinetobacter* spp.) by the World Health Organization in 2017. The monitoring of the different anthropogenic activities (hospital, informal settlements, veterinary clinic) and the operational efficiency of the WWTP along the Umhlangane river, were essential to determine the possibility of the dissemination of antibiotic-resistant bacteria and the genes associated with resistance and virulence into the environment. The different activities (hospital, veterinary hospital, informal settlement, and WWTP) that occur along the river made it a suitable study site. The aim of this study was, therefore, to isolate and characterise the selected bacteria from wastewater and surface water samples and to determine their antibiogram, ARG, and VG profiles as well as to develop rapid advanced methods for detection of the selected target bacteria from the environmental samples. This was achieved through three comprehensive objectives and the summary of the findings follow below:

### 7.1.1 Objective 1

The purpose of this objective was to isolate the bacteria of interest from the various sampling sites and to also determine the concentration of these bacteria. Wastewater and surface water samples from the different sites were analysed for *Acinetobacter* spp., *Aeromonas* spp., *Pseudomonas* spp., and *Stenotrophomonas maltophilia*. The average plate counts ($\log_{10}$ CFU/mL) in the influent and effluent of the selected bacterial species were as follows: *Aeromonas* spp. (6.27±0.25 and 2.91±1.46, respectively); *Pseudomonas* spp. (3.19±1.46 and 1.72±1.40, respectively); *Acinetobacter* spp. (5.30±0.9 and 3.30±0.71, respectively) and *Stenotrophomonas maltophilia* (4.7±0.9 and 3.4±0.71 respectively). Among the surface water samples analysed, the veterinary site had the highest impact on the concentration of bacteria in the river as there was an increase in the bacterial concentration of 0.6 $\log_{10}$ CFU/mL compared

to upstream samples. Similarly, at the hospital site, higher concentrations of *Aeromonas* spp. (3.1±0.7 $\log_{10}$ CFU/mL) and *Pseudomonas* spp. (1.2±1.4 $\log_{10}$ CFU/mL) were found in downstream samples. This could be due to stormwater drainage points from the hospital and veterinary clinic, which was located close to the downstream sampling points. In surface water samples *Acinetobacter* spp. had the highest concentration (2.6±1.2 $\log_{10}$ CFU/mL) reported upstream of the WWTP effluent discharge point. *S. maltophilia* was detected less frequently in the water samples and the highest concentration was recorded for this potential pathogen at the sampling point upstream of the wastewater discharge point. On investigation of the roots of plants that grow along the river and river sediments, higher concentrations of *S. maltophilia* were found. The characterisation of the isolates was performed using PCR and MALDI-TOF MS. The results indicated that *Pseudomonas* isolates comprised of the following species *P. putida* (51%), *P. aeruginosa* (26%), *P. mendocina* (16%), *P. alcaligenes* (5%), and *P. oleovorans* (2%). The isolates belonging to the genus *Aeromonas* were speciated and comprised of *A. hydrophila/caviae* (58%), *A. caviae* (13%), *A. hydrophila/caviae/sobria* (11%), *Aeromonas* spp. (8 %) and *A. veronii/sobria* (10%). The MALDI-TOF MS identification showed that out of the 38 PCR positive *Acinetobacter* isolates, 61% of the isolates were unknown *Acinetobacter* spp. and the others were, *Acinetobacter haemolyticus* (3%), *Acinetobacter baumannii* complex (26%) and 5% each were *Acinetobacter johnsonii* and *Acinetobacter lwoffi*. All 25 *S. maltophilia* isolates were confirmed by PCR and MALDI-TOF MS. From this investigation, it can be noted that a concise MALDI-TOF MS library database is imperative for the accurate identification of bacteria.

### 7.1.2 Objective 2

The focus of this objective was to determine the antibiotic resistance and virulence gene profiles of the isolated target bacteria using both phenotypic and genotypic methods. Antibiotic susceptibility testing was performed on all four selected target bacteria against 12 antibiotics.

The antibiogram of *Aeromonas* (*n*=59) showed that the isolates were resistant against trimethoprim-sulphamethoxazole (100% of isolates), ampicillin (76%), polymyxin B (56%), and colistin (42%). Lower levels of resistance were displayed towards the fluoroquinolones, carbapenems, and third-generation cephalosporins. Although 74% of *Aeromonas* isolates were resistant to ampicillin, the $bla_{AmpC}$ genes were detected in only 8% of the isolates. *Aeromonas*

isolates had higher MAR indices at the hospital site. The pathogenicity of *Aeromonas* spp. is multifaceted and involves various factors which act together or independently of each other at different stages of infection. The aerolysin gene (*aer*) which has haemolytic, cytotoxic, and enterotoxin activity when expressed, was found in 31% of *Aeromonas* isolates. The flagellin gene responsible for flagella production was found in 25% of isolates. There was a positive and significant association of ARG, RP, and VG and this indicates that the isolates can be both pathogenic and resistant to antibiotics.

*Pseudomonas* isolates exhibited resistance against the carbapenems; meropenem (21% of isolates) and imipenem (7%), this is a concern as the WHO has listed *Pseudomonas aeruginosa* as a priority bacterium with the need for new antibiotics. *Pseudomonas* also displayed increased resistance againstwards ampicillin (93%) and cefixime (95%). *Pseudomonas* isolates were highly sensitive to traditional antibiotics such as fluoroquinolones and polypeptide (colistin). The most prevalent putative gene detected from *Pseudomonas* spp. was *exoS* (26%), which is responsible for cytotoxin production, and this contributes significantly to pathogenesis.

*Stenotrophomonas maltophilia* has emerged as an important opportunistic pathogen, which causes infections that are often difficult to manage. Alarmingly, *S. maltophilia* (*n*=25) isolates showed absolute resistance against the sulphonamide (trimethoprim-sulphamethoxazole), the carbapenems (imipenem and meropenem), and third-generation cephalosporin's (cefixime). *S. maltophilia* (100%) isolates (irrespective of the sample matrix) had MAR indices greater than 0.2. *S. maltophilia* isolates downstream of the hospital and upstream of the WWTP had MAR indices of 0.8 and 0.7 respectively. *S. maltophilia* isolates from the sediment and plant rhizosphere had lower MAR indices than the isolates from water, although MDR was less frequent in the isolates from water. Although all *S. maltophilia* isolates were resistant against trimethoprim-sulphamethoxazole, *sul1* genes were present in only 8% of the isolates. All *S. maltophilia* isolates overexpressed the multidrug efflux pumps (s$me_{ABC}$, $sme_{DEF}$) which are known to contribute to intrinsic MDR.

*Acinetobacter* (*n*=38) isolates were resistant to trimethoprim-sulphamethoxazole (96%), polymyxins (*i.e.*, polymyxin B [86%] and colistin [42%]). Isolates in this study were of concern as they were found to be resistant to the carbapenems; imipenem (4%) and meropenem (19%) and carbapenem-resistant *Acinetobacter baumannii* have been listed by the WHO as a priority

bacterium that urgently requires new antibiotics. Of the *Acinetobacter* isolates, 78.9% had MAR indices that were greater than 0.2. The highest MAR indices for the *Acinetobacter* isolates were recorded in isolates from the hospital downstream (0.4) and upstream of the WWTP (0.5). *The* ARG, *sul1*, and *sul2* were found in 24% and 29% of *Acinetobacter* isolates respectively. *blaOXA 23/51* was found in 21% of *Acinetobacter* isolates and 29% of the isolates contained the $bla_{TEM}$ gene.

### 7.1.3 Objective 3

This objective evaluated the application of two rapid and advanced methods (FCM and ddPCR) for the detection and quantification of the selected target bacteria from wastewater influent and effluent in comparison to conventional plate count methods that are predominantly used. The total plate counts for the wastewater influent and effluent samples averaged at 6.46±0.02 and 4.63±0.7 ($log_{10}$ CFU/mL), respectively. The detection of each of the target bacteria using selective plate counts was lower in concentration than FCM and ddPCR. Overall, the highest concentration of selected target bacteria was detected with ddPCR analysis. Although the ddPCR method did not allow the differentiation of DNA between live vs. dead bacteria in this instance, the method has the potential to replace the traditional plate count method. The BacLight kit was used with FCM to determine the viable counts of the wastewater samples and the total viable count ($log_{10}$ bacteria/mL) ranged between 8.00±0.02 - 7.72±0.05 (influent) and 6.84±0.11 - 6.60±0.04 (effluent) which was higher than the counts measured with the conventional plate count method. The Flow-FISH approach was used to determine the concentration of each of the selected target bacteria using FCM, due to its ability to account for both viable and VBNC bacteria. With Flow-FISH analysis selected target bacterial counts were detected in the higher concentration than plate counts (Table 6.2.) and this proves that culture methods can be limited as cells can develop a VBNC state whereby they are in an active form but are unable to grow on culture media. The investigations into the use of the different culture-dependent and independent methods show that the methods are complementary and can be used together to gain an understanding of bacterial communities in biological samples such as wastewater. However, for routine investigative use, the culture-independent methods may be more feasible due to the ability to produce results more timeously. Due to the lack of success with some culture methods to account for VBNC and as ddPCR analysis in this instance detected non-viable DNA as well, FCM techniques such as viability studies and the combination of techniques such a Flow-FISH

seems to be an efficient strategy for the detection and the quantification of viable cells, including those in a VBNC state.

## 7.2 Major Conclusion

Untreated wastewater and surface water contain a high concentration of *Aeromonas*, *Pseudomonas*, and *Acinetobacter*, however *S. maltophilia* was not easily detected from water samples using conventional methods. *S. maltophilia* was however detected in plant rhizospheres and sediments. The treatment process was efficient in decreasing the concentrations of selected target bacteria. A trend that was noticed, was the impact of the anthropogenic activities along the river, especially at the hospital and veterinary site, where MAR indices were increased. Many of the target bacteria were MDR and contained ARG and VG. This is of concern as these genes could be transferred to the natural microorganisms found in the surface water body. Although conventional plate count methods have been used in the first two objectives, the application of rapid methods such as ddPCR and FCM may be more feasible for more frequent use, and for an efficient outcome, Flow-FISH would be practical.

## 7.3 Recommendations

- There is a need for the monitoring of additional WWTPs and receiving water for the selected target bacteria that is part of this study from other locations in South Africa to understand their prevalence in the country.

- It is recommended that rapid and advanced methods such as Flow -FISH, and ddPCR be used in parallel with traditional culturing methods. These methods are specific and FCM can detect VBNC bacteria to ensure correct enumeration and ddPCR can detect bacteria present in low concentrations.

- It would be recommended to perform a complete metagenomic study of this area to understand the complete profile of emerging pathogens.

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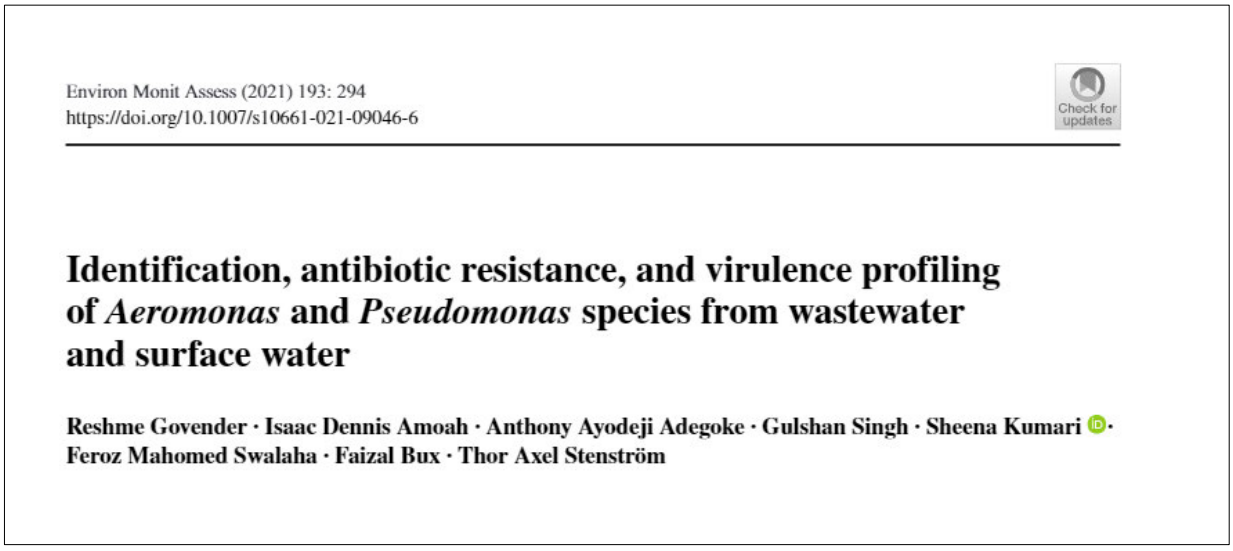
# Appendices

## Appendix A

### Publications



Environ Monit Assess (2021) 193: 294
https://doi.org/10.1007/s10661-021-09046-6

Check for updates

**Identification, antibiotic resistance, and virulence profiling of *Aeromonas* and *Pseudomonas* species from wastewater and surface water**

**Reshme Govender · Isaac Dennis Amoah · Anthony Ayodeji Adegoke · Gulshan Singh · Sheena Kumari · Feroz Mahomed Swalaha · Faizal Bux · Thor Axel Stenström**

Taylor & Francis

Journal of Environmental Science and Health, Part A
Toxic/Hazardous Substances and Environmental Engineering

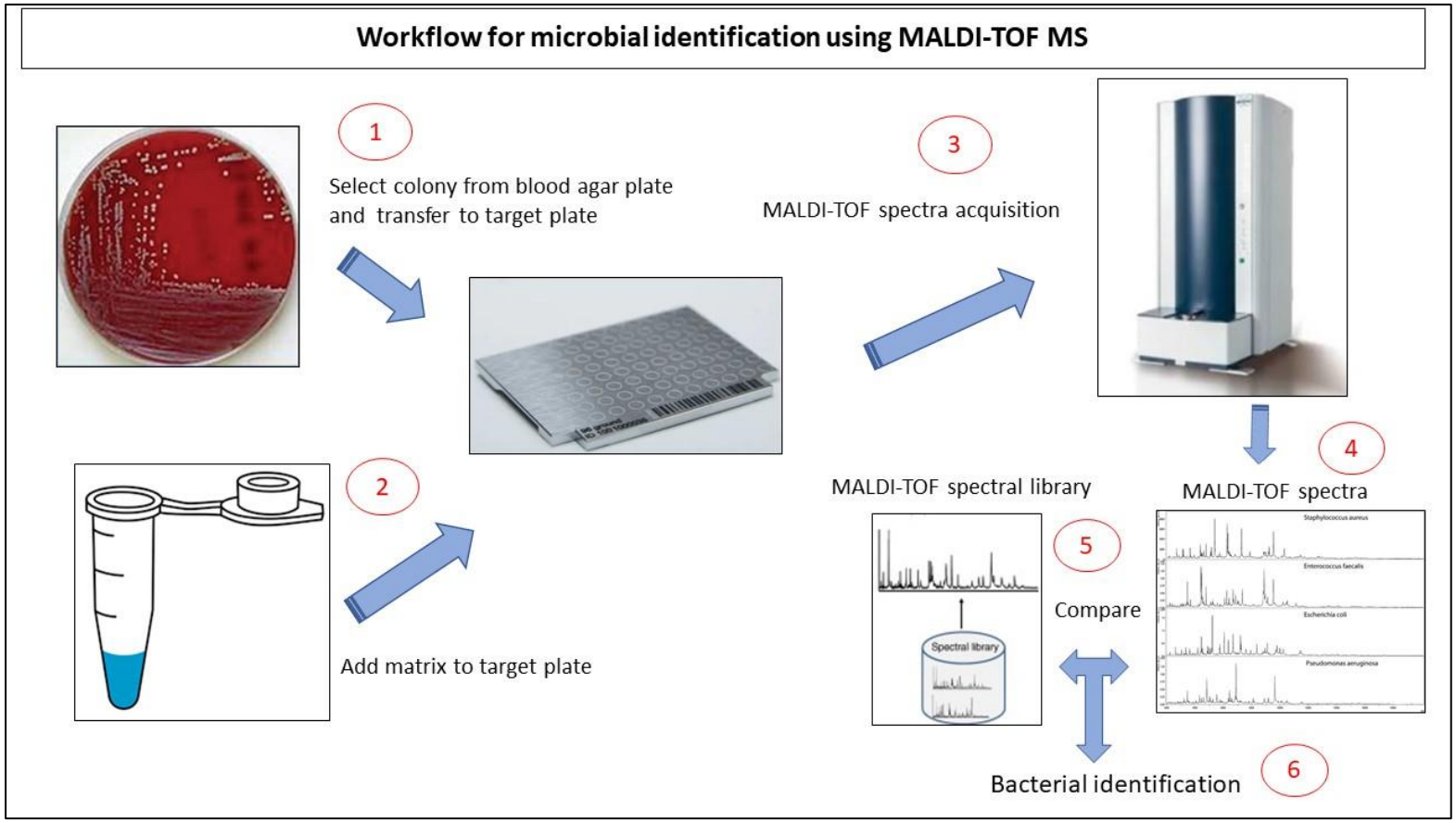
ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/lesa20

**Detection of multidrug resistant environmental isolates of *acinetobacter* and *Stenotrophomonas maltophilia*: a possible threat for community acquired infections?**

Reshme Govender, Isaac D Amoah, Sheena Kumari, Faizal Bux & Thor A Stenström

# Appendix B

## Schematic illustration of the MALDI-TOF MS workflow



# Appendix C

## Schematic illustration of the antibiotic resistance profiling workflow