Microplastic-Associated Biofilms as Reservoirs for *Vibrio* spp. and *tetB* in Urban Rivers of Eastern Cape, South Africa: The Role of Microplastics in the Enrichment and Persistence of Pathogens and Antibiotic Resistance Genes

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ABSTRACT

Microplastic (MP) pollution has become a significant concern, as these small particles can persist, accumulate and contribute to the persistence and spread of waterborne pathogens such as *Vibrio* spp. and antibiotic resistance genes (ARGs) such as *tetB* in freshwater systems. MPs can originate from intentional production (primary MPs) or the weathering of larger plastics (secondary MPs). In South Africa, the interconnected issues of plastic pollution, water quality management and microbial contamination of water sources remain insufficiently addressed, especially regarding microplastics which can carry pathogens and antibiotic resistance genes (ARGs) and may pose significant risks to environmental and public health. Amidst the rising cholera and vibriosis incidences in South Africa, this study assessed the role of MP biofilms in the enrichment of *Vibrio* spp. and *tetB* in the Kat and Swartkops rivers, Eastern Cape, South Africa, and thus their potential role in persistence of antibiotic resistance and waterborne infections. A literature review was conducted to examine MP pollution in aquatic environments, MP role in harbouring antibiotic-resistant bacteria (ARB) and ARGs, including *Vibrio* spp. and *tetB*, as well as to elucidate methodological approaches for analysing MP biofilm communities and ARGs in rivers. Thereafter, water and MPs were sampled from ten sites along the Kat and Swartkops rivers impacted by various land uses in March and July 2023. Concurrently, physicochemical parameters of the river water including temperature, salinity, dissolved oxygen, turbidity, nitrates, nitrites, phosphates and ammonia were measured to assess their potential as drivers of *Vibrio* and *tetB* in these environments. Microplastic particles were visualised using stereo microscopy, counted and classified into four groups based on their shape. Novel primers targeting the *recA* gene were designed and evaluated in-silico and experimentally for enhanced detection of *Vibrio* directly from river water samples. A comparative analysis of this primer with the previously published and widely used *Vibrio* spp. *16S rRNA* primer (V.16S-700F/1325R) was conducted to assess their relative specificity, sensitivity and amplification efficiency. The primer with superior performance was thereafter used for Quantitative Polymerase Chain Reaction (qPCR) to measure the relative abundance of *Vibrio* spp. *16S rRNA* and *tetB* genes. A comparative C_T method ($2^{-\Delta CT}$) was used to calculate the relative abundance of *Vibrio* spp. *16S rRNA* and *tetB* per total bacteria (via *16S rRNA* gene) in water and MP biofilms. Lastly, findings from the quantitative

assessments of *Vibrio* spp. and *tetB* gene prevalence were analysed in relation to water physicochemical parameters.

The MP abundance ranged from 0.69 to 4.03 particles/L with a mean of 1.52 particles/L in the Swartkops River, and 0.74 to 2.20 particles/L with a mean of 1.55 particles/L in the Kat River. MP fragments were the most abundant type of microplastic, followed by fibres, films and pellets. In both rivers, the sites downstream of wastewater treatment effluent discharge points showed the highest MP abundance. Compared to the primer designed in this study, the *16S rRNA* primer demonstrated superior performance, achieving a higher sensitivity (LOD = 10^3 gene copies/µL). For amplification efficiency, positive signals were observed between 10^7 to 10^3 gene copies/µL, yielding a regression coefficient (R^2) of 0.999, a slope of -3.75, and an efficiency of 85%. Melt curve analysis revealed a single peak at 88.16 ± 0.15°C, confirming amplification specificity. In contrast, the *recA* primer exhibited a lower sensitivity (10^4 gene copies/µL) and slightly lower detection rates of *Vibrio* spp., at 95% of river water samples and 42% of biofilm samples, compared to 100% detection in water and MP biofilms by the *16S rRNA* primer.

The relative abundance of *Vibrio 16S rRNA* ranged from 9.46 x 10^{-3} to 1.86 copies/*16S rRNA* in water and 4.66 x 10^{-3} to 0.82 copies/*16S rRNA* in MP biofilms. In contrast, *tetB* relative abundance ranged from 1.60 x 10^{-8} to 1.55 x 10^{-4} copies/*16S rRNA* in water and 9.51 x 10^{-8} to 8.46 x 10^{-4} copies/*16S rRNA* in MP biofilms. However, there was no significant difference in the gene abundances between water and the MP biofilm (Student t-test, $p > 0.05$). Spearman's rank correlation analysis revealed moderate correlations between river physicochemical conditions and *Vibrio 16S rRNA* and *tetB* in both rivers. Additionally, land use factors such as wastewater treatment discharge, agricultural runoff and industrial effluents were found to influence *Vibrio* spp. and *tetB* abundances. These findings do not support the assumption that MPs enrich bacterial pathogens and their ARGs, including *Vibrio* and *tetB*. Instead, the study identifies anthropogenic sources, particularly wastewater treatment works (WWTW), as a primary driver of bacterial and ARG abundance in urban rivers. These findings call for improved WWTW infrastructure to mitigate microplastic contamination and the proliferation of ARB and ARGs in urban rivers. Significantly, there is a need for interventions that consider multiple river pollution sources, instead of focusing solely on MPs to mitigate ARB and ARG dissemination.

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ABBREVIATIONS AND ACRONYMS

PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
ARB	Antibiotic Resistant Bacteria
WWTW	Wastewater Treatment Works
ARG	Antibiotic Resistance Gene
TRG	Tetracycline Resistance Gene
BMRG	Biocide/Metal Resistance Gene
MP	Microplastic
PP	Polypropylene
PE	Polyethene
PET	Polyethylene terephthalate
PS	Polystyrene
PVC	Polyvinyl Chloride
PA	Polyamide
PMMA	Polymethyl methlylacrylate
PHB	Polyhydroxybutyrate
NGS	Next-generation sequencing
FTIR	Fourier Transformed Infrared Spectroscopy
ATR-FTIR	Attenuated Total Reflectance - FTIR
MGE	Mobile Genetic Element
VF	Virulence Factor
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
HGT	Horizontal Gene Transfer
VBNC	Viable but Non-Culturable
WHO	World Health Organization
UNEP	United Nations Environmental Programme
RSA	Republic of South Africa
NWA	National Water Act
NWRS	National Water Resource Strategy
NEMA	National Environmental Management Act

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DEDICATION

This thesis is dedicated to my mother Gladys Nnadozie. May you reap the fruit of your labour of love.

CHAPTER 1: BACKGROUND

1.1 THE GLOBAL PLASTIC POLLUTION CRISIS

The continuous increase in the production and use of plastics, coupled with inadequate waste management practices, has led to widespread plastic pollution in aquatic environments. Global plastic production reached 460 million tonnes in 2019, with approximately 353 million tonnes of plastic waste generated annually (OECD, 2022). However, only 28% of plastic waste is recycled or disposed of sustainably, while the remaining 72% accumulates in the environment (Vermeiren, Muñoz and Ikejima, 2016; Verster *et al.*, 2020). According to one estimate, 19–23 million tonnes of plastic waste enter the world's aquatic ecosystems annually (UNEP, 2024), breaking into fragments of different sizes and classifications, such as nano (1–1000 nm), micro (1–1000 µm), meso (1–10 mm) and macro (> 1 cm) plastics (Bermúdez and Swarzenski, 2021). Among these fragments, microplastics (MPs) have received much attention. These particles originate from various sources such as textiles, personal care products, plastic packaging and even tyre wear (Duis and Coors, 2016; Liu *et al.*, 2020; Song, Wang and Li, 2024). They are transported into the rivers via surface runoff, sewage systems and wastewater treatment plant effluents (Lee *et al.*, 2024). Microplastics have been found in several urban rivers globally, such as the Pearl River, China (L. Lin *et al.*, 2018), the rivers Trent, Soar and Leen in the United Kingdom (Stanton *et al.*, 2020), the Arakawa and Tone Rivers in Japan (Guruge *et al.*, 2024), the Mississippi and Chicago rivers, USA (McCormick et al., 2014; Wontor, Olubusoye and Cizdziel, 2024, and the Rhine and Danube, Europe (Lechner *et al.*, 2014; Mani *et al.*, 2015). Globally, MPs have emerged as a significant pollutant of concern in aquatic environments, due to their ubiquity and persistence (Ali *et al.*, 2024). Once introduced into aquatic habitats, they persist due to their durability and resistance to degradation, posing a significant threat to aquatic ecosystems (Geyer, Jambeck and Law, 2017; Zimmermann *et al.*, 2021). Microplastic debris has been implicated in the mortality of various aquatic organisms. Ingestion of plastics mistaken for food has caused obstruction and physical damage in the digestive system of fish and crustaceans (Zhang *et al.*, 2023; Khan *et al.*, 2024). In addition, their surface

hydrophobicity allows MPs to adsorb various chemicals including heavy metals, chemical additives and persistent organic pollutants, leading to contamination of the natural environment where they come to rest (Chen *et al.*, 2024; Tenea *et al.*, 2024). Furthermore, plastic additives such as synthetic dyes, bisphenol A, phthalates, and perfluorinated compounds, commonly used to make plastics water-repellent, are carcinogenic, disrupt endocrine function, and harm fertility and brain development in humans (Maddela *et al.*, 2023). These additives and chemicals that have been adsorbed may leach out of MPs and enter the tissues of aquatic organisms which consume these particles, including those consumed as seafood by humans (Campanale, Massarelli, *et al.*, 2020).

1.2 THE CHALLENGE OF AQUATIC PLASTIC POLLUTION AND WATER QUALITY MANAGEMENT IN SOUTH AFRICA

1.2.1 Level of Plastic Pollution in South Africa and Drivers

The plastic industry in South Africa is one of the largest, with an estimated 1.2 million metric tons produced in 2021 alone (Mokgalaka-Fleischmann *et al.*, 2024). South Africa is estimated to be among the top 20 plastic producers in the world, and among the top 3 in Africa contributing to aquatic plastic litter each year (Jambeck *et al.*, 2018; Verster *et al.*, 2020; WWF, 2022). The high use and improper disposal of these plastics leads to the indiscriminate accumulation of plastic waste and pollution of aquatic environments, especially in freshwater. Emerging evidence suggests that South Africa's rivers are polluted with MPs. Microplastics have been detected in the Vaal, Crocodile, Mooi, Mvudi and Plankenburg rivers in South Africa (Bouwman *et al.*, 2018; Dahms, van Rensburg and Greenfield, 2020; Dalu *et al.*, 2021; Apetogbor *et al.*, 2023; Saad *et al.*, 2024). The main causes and drivers of plastic pollution in aquatic ecosystems are related to human population growth, urbanization, industrialization, and municipal wastewater treatment works that are operated beyond design capacity and poorly maintained (Nel *et al.*, 2017; Viljoen and Van Der Walt, 2018; Akindele and Alimba, 2021). In addition, many treatment plants discharge wastewater that still contains significant levels of enteric pathogens, contaminating downstream water bodies and posing serious public health risks (Osuolale and Okoh, 2017; Mishra *et al.*,

2023). Enteric bacteria, including *Vibrio* spp., demonstrate resilience in wastewater, surviving conventional treatment processes and even developing resistance to disinfectants such as chlorine (Okeyo *et al.*, 2018).

1.2.2 Water Quality Management: Policies, Challenges, and Gaps

South Africa boasts a remarkable legal framework to protect water resources and guarantee environmental sustainability. The country's Constitution (Act 108 of 1996) is one of the key policies and enshrines the right of all South Africans to an environment that does not threaten their health or well-being. Additionally, it orders environmental protection to benefit both present and future generations. The Constitution allocates roles to the national government, which has authority over freshwater resources, and to municipalities, which are tasked with administering water services (potable water supply and sanitation). Local governments are responsible for managing and prioritizing basic water needs for communities and supporting social and economic development (RSA, 1996).

The National Water Act (NWA) further contributes to the sustainable and equitable management of water resources, placing the national government as the public trustee of water. The Minister of Water and Sanitation is responsible for managing water in a way that addresses equity, redress, efficiency and safety (NWA No. 36 of 1998; RSA, 1998). The Act's National Water Resource Strategy (NWRS) sets the strategic direction for water management and is reviewed every five years. The Act defines a Reserve to ensure that water is set aside for basic human needs and to protect aquatic ecosystems. The Act also introduces a water resource classification system to regulate the use of different water resources and mandates water use licenses, regulating activities such as water extraction, storage and alteration of watercourses. Additionally, financial measures are in place to promote efficient management of water resources (NWA No. 36 of 1998; RSA, 1998; DWS, 2022).

The Water Services Act (WSA) is specifically concerned with the delivery of water and sanitation services to communities, ensuring that everyone has access to the basic water supply necessary to maintain health and hygiene. The Act acknowledges the role of local government in providing water services and tasks them with ensuring affordable, efficient and sustainable access to water. The national government supports municipalities in this role, and the Act also sets up frameworks for water

services institutions to manage the provision of these services (WSA No. 108 of 1997; RSA, 1997).

The National Environmental Management Act (NEMA) provides a framework for environmental governance in South Africa, ensuring compliance with the constitutional environmental right. It advocates for sustainable development, environmental justice and public participation. For water resources, NEMA aligns environmental licensing with water use authorizations issued under the NWA, promoting integrated management of environmental and water resources. This ensures that water management is carried out in a coordinated and responsible manner (NEMA No. 107 of 1998; RSA, 2024).

Despite these comprehensive regulations, challenges persist in effectively managing water quality. Issues such as plastic pollution, inadequate wastewater treatment, insufficient enforcement and rapid population growth continue to strain the protection of water resources in South Africa (Odume, 2022).

1.3 THE MICROPLASTISPHERE AS A RESERVOIR FOR PATHOGENS AND ANTIBIOTIC RESISTANCE GENES (ARGS)

Microplastic (MP) pollution not only poses a significant threat to aquatic ecosystems but also provides an environment conducive for the proliferation of diverse microbial communities through biofilm formation. The high surface area and chemical properties of MPs make them ideal habitats for microbial colonization, forming what is known as the ‘microplastisphere’ (Zettler, Mincer and Amaral-Zettler, 2013). The microorganisms within the microplastisphere, including pathogenic bacteria, have gained attention in recent years due to their potential risks, especially in environmental public health (Junaid, Liu, *et al.*, 2022). These biofilms not only provide a stable environment for bacteria but also facilitate the horizontal transfer of genetic material, including antibiotic resistance genes (ARGs) (Pham, Clark and Li, 2021; Junaid, Liu, *et al.*, 2022).

Pathogenic bacteria such *Vibrio* spp. have frequently been reported in the aquatic microplastisphere (McCormick *et al.*, 2014; Laverty *et al.*, 2020; Wang *et al.*, 2021; Q. Li *et al.*, 2022; Xiao *et al.*, 2023; Guruge *et al.*, 2024). *Vibrio* species are naturally found in a wide range of aquatic environments, including both freshwater and marine systems. However, the detection of certain pathogenic species in freshwater habitats,

where they were previously rarely reported, is becoming increasingly common, likely driven by anthropogenic pollution and rising water temperatures (Brumfield *et al.*, 2021; Nigro *et al.*, 2022). *Vibrio* spp. are gram-negative bacteria that include species such as the widely known *V. cholerae* which cause human infections. *Vibrio cholerae* is the main agent that causes cholera, a severe diarrheal disease spread through contaminated food or water. It remains a global public threat and is an ongoing pandemic especially prevalent in regions with compromised water, sanitation and hygiene (WASH) infrastructure (Usmani *et al.*, 2021). There are also other pathogenic *Vibrio* species such as *V. parahaemolyticus* and *V. vulnificus* which pose significant public health risks. In the United States, about 80,000 illnesses and hundreds of deaths are caused by *Vibrio* spp. annually, and a significant proportion of waterborne deaths are caused by *V. vulnificus* (Newton *et al.*, 2012; Baker-Austin and Oliver, 2018; CDC, 2024). Pathogenic and non-pathogenic *Vibrio* strains have been found on MPs. Some studies have found that *Vibrio* abundance on MP biofilms exceeds that than in the surrounding aquatic environment (Kirstein *et al.*, 2016; Frère and al., 2018). These studies have evaluated different plastic types and forms, showing that *Vibrio* spp. often colonize polyethylene (PE), polypropylene (PP), polystyrene (PS) and polyvinyl chloride (PVC) particles, including fragments, films, fibres and microbeads.

Furthermore, the microplastisphere accumulates several classes of ARGs including tetracycline, sulphonamide, aminoglycoside and quinolone resistance genes, mobile genetic elements such as integrons and integrases, and plasmids, especially in freshwater environments (J. Wang *et al.*, 2020; S. Wang *et al.*, 2020; Wang *et al.*, 2021; Junaid, Liu, *et al.*, 2022; Xu *et al.*, 2022; Zhou *et al.*, 2022). Tetracycline-resistant bacteria have emerged in aquatic habitats due to the widespread use of tetracycline (Tc) in humans and animals (Obayashi *et al.*, 2020). Among the more than 38 determinants that contribute to Tc resistance in *Vibrio* species, *tetB* is one of the most frequently documented in environmental isolates (Kim *et al.*, 2007; Jahantigh *et al.*, 2020). This gene encodes efflux pumps that export Tc from bacterial cells, conferring resistance (Chopra and Roberts, 2001). Their presence increases the risk of these environments becoming reservoirs of tetracycline resistance genes (TRGs) (Jahantigh *et al.*, 2020; Otokunefor *et al.*, 2023). ARGs carried by pathogenic bacteria reduce the effectiveness of antibiotic treatments, resulting in infections that are more difficult to treat and pose a serious threat to human health (Murray *et al.*, 2022). The various studies mentioned in this chapter have proven that microplastics can harbour

pathogenic *Vibrio* species and ARGs and suggest that these MPs are concentrating/enrichment carriers. The presence of *Vibrio* spp. and their ARGs on MPs is particularly concerning due to their association with cholera and other diarrhoeal diseases.

1.4 EPIDEMIOLOGY OF CHOLERA AND *VIBRIO*-RELATED INFECTIONS IN SOUTH AFRICA

South Africa has experienced multiple cholera outbreaks, with recorded cases dating back as far as the 1980s. One of the most severe epidemics occurred in 2000–2001, resulting in over 106,000 infections and 232 deaths nationwide. In 2003, about 3901 cases were reported in South Africa, with 45 deaths as the outbreak spread to Kwazulu-Natal, Mpumalanga, Gauteng and the Northern Province before subsiding (Le Roux, 2004).

The Eastern Cape Province has experienced recurring outbreaks. In 2004, 738 cases of cholera were diagnosed, with four deaths. Another outbreak in the Eastern Cape, specifically in the Greater Barkly East Area of Ukhahlamba District Municipality occurred in 2007, resulting in 80 child deaths (Bateman, 2009). Cholera was officially declared endemic by the South African Ministry of Health in 2009 due to these persistent outbreaks in the Eastern Cape and other provinces, particularly Mpumalanga's Nkomazi, Kwazulu-Natal and the North West Province (The Mail & Guardian, 2009).

Between February and June 2023, a cholera outbreak across 5 provinces including Gauteng, Free State, Limpopo, Mpumalanga and North West, resulted in 166 confirmed cases, with 31 deaths, the majority of which occurred in Gauteng (South African Government, 2023). The outbreak was attributed to imported strains of *Vibrio cholerae* O1 biotype El Tor (7PET), which were genetically linked to an ongoing cholera outbreak in Malawi that began in September 2022 (Smith et al., 2023; NICD, 2025).

In addition to cholera, the Eastern Cape has been particularly vulnerable to diarrheal disease outbreaks linked to poor water quality. According to the residents of Fort Beaufort and surrounding areas, the poor quality of the municipal water supply was responsible for a diarrhoea outbreak in 2014 which caused many hospitalizations and deaths (SABC Digital News, 2014). In 2015, a contaminated water-related scare

occurred in Chris Hani District Municipality in the Cradock area, although the cause of the gastroenteritis was not confirmed (Ismail, 2015). These recurring outbreaks highlight the public health risks associated with water sources in the region and their potential role as reservoirs of disease.

1.5 STUDY JUSTIFICATION

The increasing presence of MPs in South Africa's rivers raises concern about their potential to act as reservoirs for pathogenic bacteria and ARGs. MPs provide a stable environment for bacteria to colonise, enrich and be protected from adverse conditions through biofilm formation. This protection enables the pathogens to survive for extended periods in the water. Additionally, the microplastisphere accumulates several classes of ARGs, including TRGs, especially in freshwater environments. Tetracyclines are affordable, widely available, and have few side effects, and so they have been used increasingly to treat animal and human infections in recent years. This has resulted in the emergence of Tc-resistant bacteria in aquatic environments. South Africa has a history of *Vibrio*-related infections, including recurring cholera outbreaks, particularly in the Eastern Cape. Poor wastewater management, inefficient wastewater treatment plants (WWTPs) and untreated effluents further exacerbate microbial contamination in freshwater sources. While the role of MPs in harbouring *Vibrio* spp. and ARGs has been well documented in marine studies, knowledge regarding their impact in freshwater ecosystems, especially urban rivers in South Africa, is limited. South Africa is a water-scarce country which relies on freshwater resources for agricultural, industrial, commercial and domestic purposes (Mack *et al.*, 2024). The presence of MPs in rivers used for drinking water, irrigation and recreational purposes presents a public health risk as they can act as reservoirs for the persistence of infections. Interactions with urban rivers which are polluted by MPs and antibiotic-resistant *Vibrio* spp. potentially exposes communities to outbreaks of antibiotic-resistant infections (Kirstein *et al.*, 2016). Furthermore, given that MPs accumulate ARGs, causing them to persist in aquatic environments for extended periods, there is a risk of amplification and spread of resistance. This increases the likelihood of widespread, multi-drug-resistant pathogens, creating a long-term public health threat. This study is crucial because it will bridge the gap in knowledge as to whether MP-associated biofilms play a role in the persistence of *Vibrio* spp. and *tetB* genes in South

African urban rivers. This knowledge is important to safeguard public health and address the wider environmental and epidemiological effects of plastic pollution.

1.6 AIM OF THE STUDY

To assess the role of MP-associated biofilms in the enrichment of *Vibrio* spp. and *tetB* in urban rivers of Eastern Cape, South Africa, and thus their potential role in the persistence of antibiotic resistance and waterborne infections.

1.7 RESEARCH OBJECTIVES

1. To investigate existing literature on ARB and ARG, including *Vibrio* spp. and *tetB* enrichment on MP in aquatic environments.
2. To assess the contamination levels of MP debris in selected urban rivers of Eastern Cape, South Africa.
3. To detect, quantify and compare the abundance of *Vibrio* spp. and *tetB* in MP-associated biofilms and surrounding water.

1.8 THESIS STRUCTURE

This thesis comprises six chapters. Chapter 1 contains a general introduction and rationale for the study. Chapter 2 provides an in-depth literature review of MP pollution in aquatic environments, its role in harbouring ARB and ARGs such as *Vibrio* spp. and *tetB* and informs methodological approaches for analysing the aquatic MP biofilm community and antibiotic resistance, including *Vibrio* spp. and *tetB*. Chapter 3 describes the general methods utilised in this study. Chapter 4 describes the approach used to develop and validate a novel primer targeting the *recA* gene for the detection and quantification of *Vibrio* spp. in environmental surface water and plastic biofilm samples. Chapter 5 details the analysis of the relative abundance of *Vibrio* spp. and *tetB* in water and MP biofilms using qPCR. Chapter 6 summarizes the work along with conclusions and recommendations for future research.

CHAPTER 2: LITERATURE REVIEW

2.1 INTRODUCTION

Microplastics (MPs; plastic particles < 5 mm) are widespread and persistent environmental contaminants (Rocha-Santos and Duarte, 2015). They may originate from intentional production (primary MPs) or weathering of larger plastics (secondary MPs). Microplastic contaminants originating from human activities now populate the globe, being found in freshwater (Z. Wang *et al.*, 2019), seawater (Aliabad, Nassiri and Kor, 2019), sediment (Martin *et al.*, 2017), soil (Garcés-Ordóñez *et al.*, 2019), air (Vianello *et al.*, 2019) and even biota (Digka *et al.*, 2024). When MPs enter aquatic environments, microorganisms attach to and colonise their surfaces to form biofilms through the secretion of extracellular polymeric substances (EPS) such as proteins, glycoproteins and glycolipids (Liu *et al.*, 2023). Microbial colonisation of plastics was first described in the 1970s when diatoms and hydroids were found attached to plastic debris in the Sargasso Sea (Carpenter and Smith, 1972). More recently, the term 'microplastisphere' was invented to denote the niche that MP particles create when various naturally occurring free-living microbial communities colonise them (Shruti, Kutralam-Muniasamy and Pérez-Guevara, 2024). Empirical evidence suggests that microplastispheres encourage microbial colonisation and biofilm formation of microbes, thus becoming vectors for pathogenic microorganisms plus accumulation of antibiotic resistance genes (ARGs) in aquatic environments (Lobelle and Cunliffe, 2011). These MPs offer an important substratum for pathogens such as *Vibrio* spp. in aquatic environments. Pathogenic *Vibrio,* including *V. cholerae, V. vulnificus* and *V. parahaemolyticus*, have been found to thrive on MPs, particularly polypropylene (PP), and these MPs act as hotspots for the enrichment of these bacteria. In several instances, the concentrations of these pathogenic bacteria on MPs have been found to exceed those noted in the surrounding water by several orders of magnitude (Zettler, Mincer and Amaral-Zettler, 2013; Laverty *et al.*, 2020; Di Pippo *et al.*, 2022). Microplastics also play a significant role in the emergence of antibiotic-resistant bacteria (ARB), multi-antibiotic-resistant bacteria (MARB) and superbugs, and can promote ARG dissemination through aquatic environments (Wang *et al.*, 2018; Lu,

Zhang, Wu, Wang, *et al.*, 2019). Microplastics, as vectors and hotspots for ARB and ARGs, contribute to horizontal gene transfer (HGT), posing significant risks to organisms, human health and the global ecosystem due to their persistence and widespread distribution. As vectors and hotspots for ARB and ARGs, MPs in aquatic environments present an environmental source of infection. Given the significance of the microplastisphere, various studies have endeavoured to quantitatively assess the enrichment of ARB and ARGs on MPs in aquatic environments by comparing them with the surrounding water column. Therefore, this chapter interrogates existing literature on ARB and ARG enrichment on MPs in aquatic environments, including *Vibrio* spp. and *tetB*. The review seeks to provide a thorough understanding of the interactions between MPs, biofilms, *Vibrio* spp., and ARGs, highlight knowledge gaps and explore the potential for MPs as vectors and reservoirs of ARBs and ARGs in aquatic environments. Additionally, this review will inform the design and methodology of this study and other future studies, offering insights into effective detection techniques and providing a comprehensive overview of the potential ecological and public health risks associated with MP contamination in aquatic ecosystems.

2.2 MICROPLASTICS IN AQUATIC ENVIRONMENTS

The concerns about MP pollution of aquatic environments are due to their persistence in the environment and activity as vectors of chemical pollutants, ARB and ARGs. Aquatic environments where these MPs have been found include rivers, lakes, seas and oceans. The data on the various aquatic ecosystems impacted by MP pollution, with concentrations observed across both freshwater systems like rivers and marine environments such as seas and oceans, are presented in Table 2-1. In rivers, based on previous studies, MP concentrations can range from 1.19–2.92 particles/m^3, while some river basins may show densities as high as 102,708–334,667 particles/m^3. This section reviews the sources, classification, and persistence of MPs in aquatic environments, focusing on their environmental impact and distribution.

2.2.1 Sources and Classification of Microplastics

Microplastic pollution primarily originates from anthropogenic sources such as agriculture, urban runoff, industrial waste and wastewater treatment plant (WWTP)

effluents (X. Yu *et al.*, 2022). In freshwater and coastal environments, wastewater discharge plays a critical role in MP distribution. For example, McCormick et al. (2014) found significantly higher concentrations of MP particles in sites downstream of a WWTP discharge point in the North Shore Channel, USA. Similarly, Li et al. (2022) observed an increasing trend in plastic pollution along the Pearl River, China, correlating with urbanization levels.

Based on shape and morphology, MPs are commonly classified into five categories: pellets, fibres, films, foams and fragments. In addition, studies frequently report a range of prevalent colours, including yellow, blue, black, red, transparent, green and white (Table 2-1).

Size-based classification of MPs also exists but varies across studies. For instance, Frère et al. (2018) categorized MP particles into three size groups: 0.3–1 mm, 1–2 mm, and 2–5 mm, while Mughini-Gras et al. (2021) used two broader size ranges: 20–100 µm and > 100–500 µm. Regarding polymer composition, MPs in aquatic environments are predominantly composed of polypropylene (PP), polyethene (PE), and polystyrene (PS), which are the most widely detected plastic types globally. It is noteworthy that the knowledge regarding microplastics size distributions in the environment is not complete, particularly regarding the smallest MPs, due to methodological limitations (Hidalgo-Ruz *et al.*, 2012; Law and Thompson, 2014; Syberg *et al.*, 2015; GESAMP, 2016).

Table 2-1. Abundance and characterization of microplastics in aquatic ecosystems

Aquatic features	**Polymer type**	**Shape**	**Size**	**Predominant Colours**	**Reference**
Rivers	PE, PP, nylon, PS, PET, PVC, PU	Fibre, sheet, fragment	1.12–6.93 mm	White, green, transparent	Guruge et al. (2024)
River	PE, PP, PB	Fragment, fibre, pellet, film	*NA*	*NA*	Hu et al. (2021)
River	PP, PE, PS, PET, PC, others	Fibre, foam, fragment, film, pellet	100 µm - 5mm	Transparent, white, yellow, black, red, green, blue	Li et al. (2022)
River	PET, PA, PMMA, PE, PP, PAN, PS, Alkyd, PVC, ABS	Fibre, fragment, film, foam	43.47–5, 765 µm	Blue, transparent, black, red	Xu et al. (2022)
River	PE, PP, PS, p-vinylpyrrolidone/vinyl acetate, cellulose	*NA*	0.14–8.62 mm	Transparent, white, yellow, red, blue	Laverty et al. (2020)
River	PBT, PE, PP, PS	*NA*	*NA*	*NA*	Xue et al. (2020)
Canal	*NA*	Fibre, fragment, pellet, foam	*NA*	*NA*	McCormick et al. (2014)

Aquatic features	**Polymer type**	**Shape**	**Size**	**Predominant Colours**	**Reference**
River	PS, PU, PA, PE, PET, PP, PVC, isoprene	*NA*	20–500 µm	*NA*	Mughini-Gras et al. (2021)
Sea	PE	Fragment	5–10 cm	*NA*	Sucato et al. (2021)
Lakes	PE, EPS, PP	Fragment, pellet, ball, film, filament	*NA*	*NA*	J. Wang *et al*. (2020)
Creeks	PE, PP	*NA*	*NA*	*NA*	J. Wang *et al*. (2020)
Mariculture system	PET	*NA*	*NA*	*NA*	Lu, Wu and Wang, (2022)
Sea	PE, PP, PS	*NA*	*NA*	*NA*	Kirstein et al., (2016)
Oceans	PS, PP, PE, PETE	*NA*	*NA*	*NA*	Amaral-Zettler et al. (2015)
Ocean	PE, PP	*NA*	*NA*	*NA*	Zettler, Mincer and Amaral-Zettler, (2013)
Mariculture system	PE	Fibre	*NA*	*NA*	Zhang et al. (2020)
Ocean	PE, PP, PS	Fragment, pellet, line, foam, film	Macro and microplastics	*NA*	Carson et al. (2013)

Aquatic features	**Polymer type**	**Shape**	**Size**	**Predominant Colours**	**Reference**
Coast	PE, PP, PS, PVC, PET, PTFE, PA, polyester	Fragment, fibre, film, others	20 μm–5 mm	Blue, brown, black, green, yellow, red, white, transparent	Bae and Yoo (2022)
Coast	PE, PP, PS, PVC, PET, PMMA, PU, silicone	*NA*	0.1–656 mm^2	*NA*	Liang et al. (2023)
Bay	PE, PP, PS	Fragment	0.3–5 mm	*NA*	Frère et al. (2018)
Estuary	PE, PP, PS	*NA*	*NA*	*NA*	Jiang et al. (2018)

**NA* indicates that the data are not available, or the variable is not measured in the published study.

2.2.2 Transport and Fate of Microplastics in Urban Rivers

Microplastics are highly persistent in the environment, enabling their transportation over long distances, a process influenced by local winds, currents, and the geographical characteristics of the coastline (Barnes *et al.*, 2009; Duis and Coors, 2016). Their density primarily determines their behaviour in aquatic environments; MPs with a higher density than freshwater (> approximately 1.0 g/cm^3) are typically submerged, while those that are less dense remain buoyant (Duis and Coors, 2016; Andrady, 2017). It is widely assumed that large rivers carry significant amounts of both macro- and microplastics to the oceans, although quantitative data on this process is limited (Klein, Worch and Knepper, 2015; Lebreton *et al.*, 2017). The transport of MPs in surface waters may differ from that of macroplastics, as smaller particles are less exposed to wind at the water's surface. Animal activity and particle aggregation may also affect the distribution of MPs (Browne, Galloway and Thompson, 2010; Long *et al.*, 2015). Besides physical movement, microplastics can undergo several forms of degradation, including biodegradation, photodegradation and mechanical abrasion, which leads to their reduction in size (Gewert, Plassmann and Macleod, 2015; Deocaris *et al.*, 2019). However, MPs' persistence suggests that they commonly remain intact over long periods and are transported over long distances. The persistence of MPs, combined with their ability to be transported by water and biological activity, leads to their widespread distribution in urban river ecosystems.

2.3 *VIBRIO* SPP. IN AQUATIC ENVIRONMENTS

This section examines the presence, environmental significance, and pathogenic potential of *Vibrio* spp. in aquatic ecosystems.

2.3.1 Overview of *Vibrio* spp. and their environmental significance

The *Vibrio* genus belongs to the family *Vibrio*naceae and encompasses gram-negative, rod-shaped, flagellated bacteria found in different aquatic ecosystems, including freshwater, brackish and marine environments. They flourish in warm waters above 17–20°C and tolerate salinities between 5 and 25 ppt, depending on the species (Vezzulli *et al.*, 2015; Brumfield *et al.*, 2021). Temperature highly influences the incidence and distribution of *Vibrio* spp. in the environment. *Vibrio* may be detected year-round in tropical waters, where there is little fluctuation in temperature. In a tropical urban estuary where temperatures are

persistently warm, between 19–31°C, *V. vulnificus* was detected throughout the year (Nigro *et al.*, 2022). In temperate regions seasonal variations are evident, with peak prevalence in summer, sustained detectability into fall (autumn in South Africa), and occasional winter persistence. In a previous study, *V. parahaemolyticus* populations stayed undetectable in winter until water temperatures surpassed 14°C, with critical thresholds at 15°C and 25°C marking increased abundance (Brumfield *et al.*, 2023). These patterns suggest an extended seasonal presence, particularly in temperate regions. Where temperature remains favourable for *Vibrio* persistence, salinity becomes a limiting factor that determines the composition of the *Vibrio* community. Different *Vibrio* species have varying salinity tolerance. For example, in a coastal area, where temperature range from 13.2 to 33°C and salinity ranges from 2.6 to 42.4 ppt, the abundance of *V. cholerae* and *V. vulnificus* was significantly associated with lower salinities, whereas *V*. *parahaemolyticus* was associated with higher salinities, with a peak concentration at a salinity of 40ppt (Diner *et al.*, 2021). During suboptimal environmental conditions, such as in winter or when there is a reduction in salinity and nutrient availability, *Vibrio* spp. enter a viable but non-culturable (VBNC) state (Sampaio *et al.*, 2022). This is a protective mechanism whereby they become metabolically inactive and cannot be cultured under standard laboratory conditions. However, they maintain their virulence potential, and when environmental conditions improve, they once again become cultivable and can still cause infections (Colwell, 2000).

In aquatic ecosystems, *Vibrio* spp. exhibit varied niche specializations (Takemura, Chien and Polz, 2014), existing as free-living bacteria or attached to biotic and abiotic surfaces, including MPs (Zettler, Mincer and Amaral-Zettler, 2013). They associate with aquatic invertebrates, including copepods, zooplankton and bivalves, which impact their distribution and transmission (Krantz, Colwell and Lovelace, 1969; Huq *et al.*, 1983; Eiston *et al.*, 2008). Filter-feeding shellfish such as oysters concentrate *Vibrio*, posing a risk to human health (Baker-Austin *et al.*, 2018).

In terms of ecological roles, *Vibrio* spp. are major players in polymer degradation, nutrient cycling and biogeochemical processes, and therefore cannot be eradicated from the environment (Pruzzo, Vezzulli and Colwell, 2008; Thompson and Polz, 2014; Le Roux and Blokesch, 2025). Pathogenic *Vibrio* spp. include *V. cholerae, V. alginolyticus., V. vulnificus, V. parahaemolyticus* and *V. mimicus.* The ingestion of food or water contaminated by pathogenic stains of *V. cholerae* results in cholera, a severe diarrhoeal disease. The non-cholera *Vibrio* spp. can cause septicaemia and Vibriosis (Baker-Austin *et al.*, 2018; Brumfield *et al.*, 2021). *V. parahaemolyticus* is a major cause of seafood-borne

gastroenteritis (CDC, 2024), and *V. vulnificus* can cause necrotizing fasciitis and septicaemia, with a fatality rate exceeding 50% (Baker-Austin and Oliver, 2018).

2.3.2 *Vibrio* spp. in urban river ecosystems

Due to the salinity, *Vibrio* spp. are typically associated with marine, coastal and brackish water environments. However, their presence and abundance in freshwater, especially urban rivers, is linked to favourable conditions such as temperature and nutrient availability. Increasing surface water temperatures due to climate change favours *Vibrio* blooms (Kokashvili *et al.*, 2015; Vezzulli *et al.*, 2015). Several previous studies have detected antibiotic-resistant *Vibrio* strains in urban rivers with high nutrient concentrations due to pollution from domestic, industrial and agricultural waste, as well as sewage and WWTW effluents (Alam *et al.*, 2014; Okeyo *et al.*, 2018; Daboul *et al.*, 2020; Bhandari *et al.*, 2023; Sacheli *et al.*, 2023).

Organic matter such as plant debris, sewage, and agricultural runoff in polluted urban rivers serves as an energy source, stimulating bacterial growth and biofilm formation. Organic material can also protect *Vibrio* from environmental stresses and facilitate their survival, particularly in harsh conditions.

Biofilm formation provides a protective environment, shielding them from antimicrobial agents, environmental stress and predation (Sampaio *et al.*, 2022). *Vibrio* spp. have been shown to colonize MP particles in urban rivers, with potential consequences for their survival, dispersal, and pathogenicity. The ability of *Vibrio* to form biofilms on microplastics enhances their persistence in urban river environments, making them a potential source of infections to humans and animals. In addition to their interaction with MPs, *Vibrio* spp. also form biofilms on various surfaces, including river sediments, rocks and submerged debris (Abe, Nomura and Suzuki, 2020).

2.3.3 Microplastics as a Reservoir for *Vibrio* spp.

Microplastics have been identified as a significant substrate for the enrichment of *Vibrio* spp. in aquatic environments (Zettler, Mincer and Amaral-Zettler, 2013). Pathogenic species like *V. cholerae, V. vulnificus and V. parahaemolyticus*, have been found on various substrates such as plastics and glass, with concentrations of these bacteria often exceeding those in surrounding water by two orders of magnitude (Laverty *et al.*, 2020). In temperate-climate estuaries studied, *Vibrio* spp. were present in all water samples and on every substrate

examined, including MPs, confirming their ability to thrive in MP biofilms (Zettler, Mincer and Amaral-Zettler, 2013; Di Pippo *et al.*, 2022).

Colonization experiments indicate that *Vibrio* concentrations increase over time, with higher numbers observed on MPs compared to other surfaces. These increases were influenced by water temperature, with warmer waters promoting higher concentrations of *Vibrio* spp. (Laverty *et al.*, 2020). This finding highlights the correlation between temperature and the abundance of *Vibrio* spp.

Several studies have corroborated the presence of *Vibrio* spp. on MPs. For example, Kirstein et al. (2016) detected *V. parahaemolyticus* on marine MPs, while *V. vulnificus* and *V. cholerae* were only observed in water samples. Zettler *et al.* (2013) reported a nearly 24% relative abundance of a *Vibrio* species on a polypropylene (PP) sample, suggesting that certain types of MPs, particularly PP, may enhance the growth and colonization of *Vibrio* spp. These findings highlight the potential role of MPs as reservoirs for the enrichment of *Vibrio* spp., including potential human and animal pathogens, posing significant ecological and public health implications.

2.4 ANTIBIOTIC RESISTANCE IN AQUATIC ENVIRONMENTS

This section discusses antibiotic resistance mechanisms in aquatic bacteria and how antibiotic-resistant genes (ARGs) spread in water systems.

2.4.1 Mechanisms of Antibiotic Resistance in Aquatic Bacteria

Antibiotic resistance genes (ARGs) have proliferated in aquatic habitats due to the continuous accumulation of antibiotics in aquatic ecosystems (Liu, Steele and Meng, 2017; Richardson and Kimura, 2020; Saima *et al.*, 2020). For example, one study found that pharmaceutical companies' antibiotic disposal practices, local industry infrastructures, and the use of antibiotics in livestock farming were point sources of antibiotics in various water bodies in China (Liu *et al.*, 2021). Selection pressures are created by the accumulation of these antibiotics over time, enriching organisms which possess resistance determinants like ARGs or are capable of degrading the antibiotics present in their surrounding environment. These selective pressures also favour the acquisition of ARGs via HGT to competent bacteria, as well as the development of novel mutations, creating a resistance phenotype (Michael-Kordatou, Karaolia and Fatta-Kassinos, 2018; Saima *et al.*, 2020; Sun *et al.*, 2020; Liu *et al.*, 2021; Valdés *et al.*, 2021). Besides antibiotics, other chemicals such as pesticides,

metals and biocides in aquatic environments further create selective pressures that facilitate the selection of ARBs and acquiring ARGs (Singer *et al.*, 2016).

Apart from selective pressure, aquatic microorganisms can create complex microbial communities, such as biofilms, where they easily share genetic material (Parrish and Fahrenfeld, 2019; Fabra *et al.*, 2021). Thus, biofilms act as repositories of resistance, making it more difficult to eliminate bacterial populations. Vertical transmission to subsequent generations, as well as HGT mechanisms, including conjugation, transduction and transformation, occur in these structured communities to transfer genetic material amongst microorganisms, with plasmids, transposons and prophages serving as genetic carriers (Michael-Kordatou, Karaolia and Fatta-Kassinos, 2018). Additionally, the transfer of genetic material in biofilms promotes the potential for mutations to occur, resulting in the formation of new ARGs. The emergence of stronger antibiotic-resistant bacterial strains can result from the continued enhancement of bacterial resistance by these novel resistance genes (Wei *et al.*, 2019).

2.4.2 Environmental dissemination of antibiotic resistance genes

HGT via conjugation is a primary mechanism for the spread of ARGs, which facilitates the development of antibiotic resistance in non-pathogenic bacteria in humans, animals and various environmental matrices (Abe, Nomura and Suzuki, 2020). Pathogenic bacteria released into the environment, for example, through agricultural runoff or sewage, acquire resistance genes from either pathogenic or non-pathogenic ARB, which serve as a reservoir and source of resistance proliferation (Larsson and Flach, 2021). Furthermore, natural selection due to high concentrations of antibiotics in the environment may contribute to the emergence of ARGs, which can then spread via direct or indirect routes to pathogenic bacteria (Saima *et al.*, 2020). Antibiotics can enter our environment in several ways, and it is known that ARB proliferate in antibiotic polluted environments due to selective pressure exerted by antibiotics on microflora in that environment. The proliferation of ARB leads to the transfer of ARGs via HGT, and resistant strains emerge and continue to evolve at these locations, which are referred to as hotspots. ARGs, ARBs, and ambient microflora, therefore, can mix at certain locations (Kunhikannan *et al.*, 2021). Humans are exposed to these ARGs in various ways, which can cause them to integrate into their microbiome (McEwen and Collignon, 2018).

Among the antibiotics of greatest concern are broad-spectrum agents such as β-lactams, fluoroquinolones, sulfonamides, and tetracyclines, which are frequently detected in aquatic

environments (Yang *et al.*, 2021). Tetracyclines, in particular, are widely used in both human and veterinary medicine, as well as in aquaculture, and have been linked to the selection and persistence of tetracycline resistance genes (TRGs) in environmental bacteria (Amangelsin *et al.*, 2023). Notably, *Vibrio* species have increasingly been found to harbour tetracycline resistance determinants, raising public health concerns due to their potential for human infection and environmental dissemination. One TRG of significance among *Vibrio* spp. is the *tetB* gene (Roberts, 2005). The *tetB* gene is widely present in *Vibrio* spp. and as such warrants focused investigation as indicators of environmental ARG burden.

2.5 *TETB* GENE AND ITS ENVIRONMENTAL SIGNIFICANCE

Tetracyclines (Tc) are a group of broad-spectrum antibiotics used against gram-positive and gram-negative bacteria (Chopra, Hawkey and Hinton, 1992). Their mode of action is via the inhibition of protein synthesis by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor (A) site (Chopra and Roberts, 2001). Tetracycline resistance usually occurs due to the acquisition of new genes often associated with either a mobile plasmid or transposon (Roberts, 1996). Tetracycline resistance genes (TRGs) are among the most prevalent ARGs in environmental bacteria (Onohuean, Agwu and Nwodo, 2022). The high rate of Tc resistance is linked to the excessive use of Tc, especially in veterinary and aquaculture environments. Tetracyclines are used as growth promoters and prophylaxis in animals such as pigs, poultry and fish (Amangelsin *et al.*, 2023). The widespread use of Tc in animals, especially in food products, has led to the consumption of Tc residue in humans through food. Additionally, Tc are used to treat a wide range of infections caused by gram-positive and gram-negative bacteria in humans (Chopra and Roberts, 2001). Tetracycline residues have been found in pharmaceutical, hospital and agricultural effluents. The high molecular stability and low metabolism of Tc in humans and animals has led to Tc pollution in the environment. About 50–80% of consumed Tc are excreted into the environment by human faeces and animal excreta because they cannot be fully absorbed and metabolized in the body (Fiaz, Zhu and Sun, 2021).

When they enter water bodies such as urban rivers, this antibiotic creates selection pressure, which promotes the dissemination of the TRGs, such as *tetB,* to other bacteria, thus increasing the abundance of ARB in the environment. This frequent exposure to Tc at sub-clinical or sub-lethal doses has led to widespread bacterial resistance to the antibiotic (Xu *et al.*, 2021). At least 34 TRGs have been described; 23 genes code for efflux pumps,

and 11 code for ribosomal protection proteins (Bokaeian *et al.*, 2014). Among these, *tetB* is the most predominant in gram-negative bacteria, especially *Vibrio* spp. (Roberts, 2005). This section outlines the functions and ecological relevance of the *tetB* gene in the context of antibiotic resistance in aquatic environments.

2.5.1 Functions and mechanisms of the *tetB* gene

The *tetB* gene is a resistance gene that encodes for efflux pumps, which are membrane-associated proteins that export Tc from the cell. This export of Tc from within the cell reduces its intracellular concentration, thus protecting the ribosomes within the cell (Han *et al.*, 2015). The *tetB* efflux is unique compared to other efflux pumps encoding TRGs because it confers resistance against Tc, minocycline and doxycycline (Roberts, 1997). The *tetB* gene has been identified in both Gram-positive bacteria such as *Bacillus* spp. and various Gram-negative species including *Escherichia coli*, *Salmonella* spp., *Neisseria*, *Pseudomonas*, and *Acinetobacter* and is recognized as the most prevalent efflux determinant among Gram-negative bacteria. While *tetB* can be located on both chromosomal and plasmid DNA, it is predominantly plasmid-borne in *Vibrio* species (Amano *et al.*, 1991; Roberts, 2005; Ekwanzala *et al.*, 2018). These plasmids frequently co-harbour genes conferring resistance to heavy metals and virulence factors such as toxins (Chopra and Roberts, 2001). The plasmid-borne nature of the *tetB* gene implies higher rates of HGT, especially among biofilm communities (Roberts, 2005). This indicates that the HGT of the *tetB* gene is of significant concern in the environment. In addition, because of this highly mobile genetic element, that is, the plasmid, that *tetB* inhabits, it is easily transferred between bacterial genera and has a wide host range.

2.6 ARB AND ARG ENRICHMENT PROCESS AND POSSIBLE MECHANISM OF FORMATION OF THE MICROPLASTISPHERE

This section explores how microplastics facilitate the enrichment of ARB and ARGs, particularly through biofilm formation.

2.6.1 Biofilm Development and Composition

Microbial species can adapt their behaviour, shifting between a free-living state and attachment to surfaces based on their physiological condition and the surrounding

environmental factors, capitalizing on the greater availability of organic material on suspended particles and surfaces (Yin *et al.*, 2019). Biofilm formation is a survival strategy which protects microorganisms from various environmental stressors. Microorganisms may settle on biological or non-biological surfaces, forming biofilms composed of a single species or a complex community including bacteria, fungi, algae and protozoa. These organisms are encased in an extracellular matrix made of polysaccharides, waste products and debris (Balcázar, Subirats and Borrego, 2015).

The microplastisphere starts developing when a planktonic cell lightly colonizes on microplastic surfaces (Kalčíková *et al.*, 2020). The rough surface of MPs offers several attachment sites for bacterial cells, enabling their colonization (Harrison *et al.*, 2014). This initial attachment is largely reversible and is mediated by weak interactions such as van der Waals forces, electrostatic attraction, hydrophobic interactions and hydrogen bonding (Flemming and Wingender, 2010; X. Yu *et al.*, 2022). Over time, microbial communities develop a stronger irreversible adhesion through molecular interactions, and through the contribution of outer membrane proteins, pili, flagella, and adhesion proteins (Lorite *et al.*, 2011) (Figure 2-1). Extracellular polymeric substances (EPSs) further promote cell aggregation, stabilizing biofilm formation while facilitating ARG retention and transfer. Over several weeks, through steady attachment, the number of living cells and EPS production increases, together with the appearance of numerous dead cells, leading to the formation of a dense three-dimensional biofilm network on MP surfaces (De Tender *et al.*, 2017). The biofilms also promote ARG persistence by offering a protective niche that shields microbes from external stressors. The enrichment of ARGs in MP biofilms begins with the initial adhesion of ARB carrying intracellular ARGs (iARGs) and the adsorption of extracellular ARGs (eARGs) (Shruti, Kutralam-Muniasamy and Pérez-Guevara, 2024). The MP biofilm improves bacterial survival, offers physical protection, and supports an elevated occurrence of genetic exchange, permitting a significantly higher abundance of ARGs in MP biofilms compared to the surrounding aquatic environment (X. Yu *et al.*, 2022).

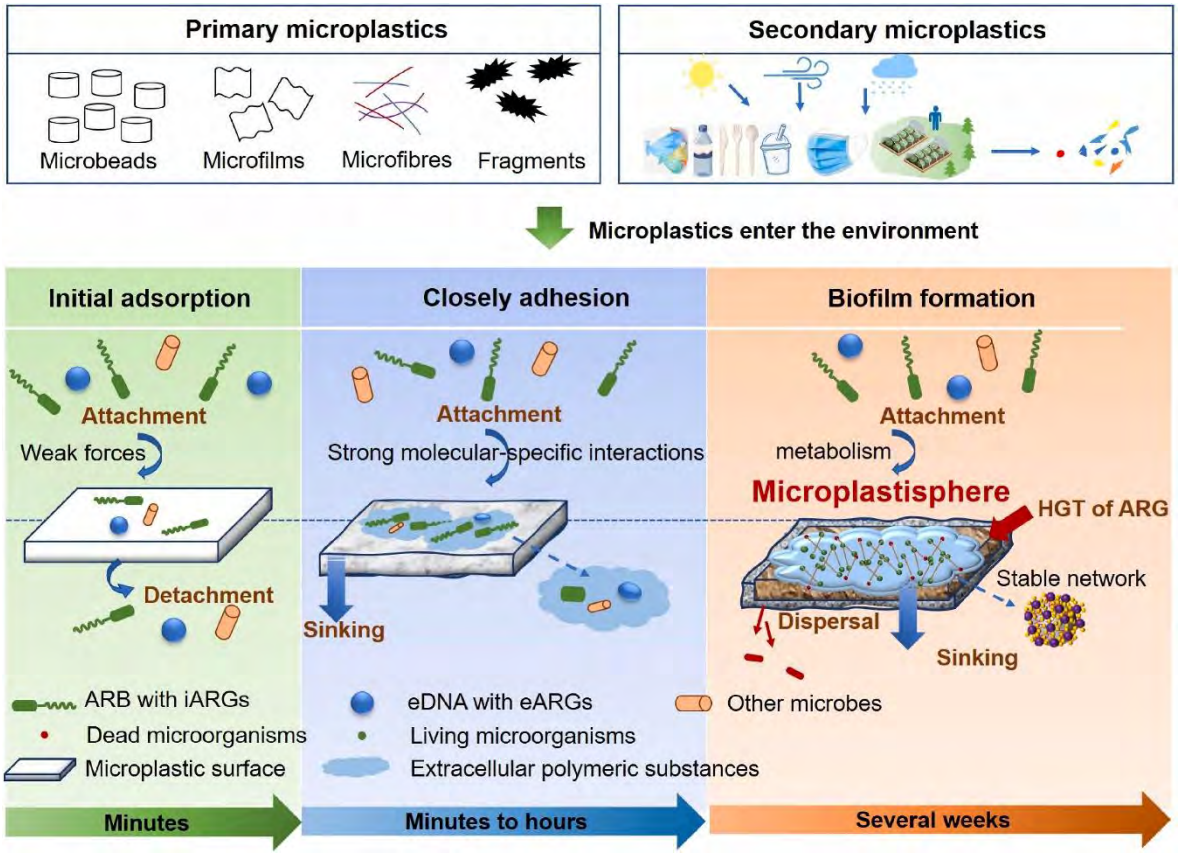

Figure 2-1. ARG enrichment process and possible mechanism with the formation of the microplastisphere. ARB: antibiotic-resistant bacteria. iARGs: intracellular ARGs. eDNA: extracellular DNA. eARGs: extracellular ARGs. HGT: horizontal gene transfer. Adapted from X. Yu et al. (2022).

2.6.2 Factors Influencing Biofilm Formation on Microplastics

Biofilm formation on MPs is influenced by environmental and physicochemical factors such as MP surface hydrophobicity, nutrient availability, pollutant type, and polymer type (Abe, Nomura and Suzuki, 2020; Ahmad *et al.*, 2024). In addition, principal aquatic conditions such as salinity, temperature, oxygen content and chemical composition are important in shaping microbial communities on MPs. Surface properties of plastics, such as roughness and hydrophobicity, affect early-stage microbial attachment, but over time, environmental conditions become the major factors in structuring biofilm communities (Rummel *et al.*, 2017). Studies show that in nutrient-poor environments, microbial colonization is more substrate-specific, showing distinguishable differences observable between plastics (e.g., PE and PS) and natural substrates (e.g., wood). On the other hand, in nutrient-rich environments such as wastewater-impacted areas, biofilm formation is less selective, and microbial communities on different substrates become more similar. This observation

implies that nutrient levels influence whether biofilm formation follows a directed, substrate-specific process or a more generalized colonization pattern (Oberbeckmann, Kreikemeyer and Labrenz, 2018). Studies suggest that salinity, temperature, oxygen availability, and dissolved organic substances released by biofilms themselves also influence colonization. However, further research is required to understand how they influence microplastic biofilm formation (Yan *et al.*, 2024). Lastly, the type of plastic colonized affects the composition of the microbial community, with distinct microbial species being selectively enriched based on the plastic's characteristics, which affects the overall diversity and structure of the biofilm. According to research by Wu et al. (2022) and Xiao et al. (2023), biodegradable plastics have higher levels of microbial diversity. Biodegradable plastics such as PLA and PHB, tend to support a more diverse microbial community than non-biodegradable plastics like PE, PP, and PET due to the greater availability of nutrients released during degradation.

2.6.3 Ecological and Environmental Implications of Microplastic-associated Biofilms

Microplastic-associated biofilms have important ecological and public health implications. For example, the transport, fate and pollutant interactions of MPs can be altered. Altering the buoyancy of the MPs by biofilms affects their mobility and deposition. This can alter their transport paths and, in certain situations, hasten their sinking, resulting in their permanent burial in sediments (He *et al.*, 2022). Also, biofilms impact microplastics' behaviour in the environment, influencing microplastics' adsorption capacity for persistent organic pollutants, heavy metals and antibiotics, which introduces new complexities in their environmental behaviour. In addition to their function in pollutant interactions, MP biofilms harbour pathogenic bacteria and toxic algae among other dangerous species that can contaminate aquatic environments and spread disease. Biofilms promote the spread of ARGs via HGT, posing a risk to human health and ecosystems (Cui *et al.*, 2023; Kumar *et al.*, 2024).

2.7 MICROPLASTICS AS RESERVOIRS AND VECTORS OF ARB AND ARGS

This section highlights the role of MPs as reservoirs and vectors for ARB and ARGs, contributing to environmental and public health risks.

Pathogenic bacteria such as *E. coli*, *Klebsiella* spp., *Pseudomonas* spp., *Campylobacter* spp. and *Vibrio* spp. have frequently been reported in the aquatic plastisphere (McCormick *et al.*, 2014; Laverty *et al.*, 2020; Wang *et al.*, 2021; Q. Li *et al.*, 2022; Xiao *et al.*, 2023; Guruge *et al.*, 2024). Additionally, several classes of ARGs, including tetracycline,

sulphonamide, aminoglycoside and quinolone resistance genes, mobile genetic elements (MGEs) e.g., integrons and integrases, and plasmids, have been found to accumulate on the plastisphere, especially in freshwater environments (J. Wang *et al.*, 2020; S. Wang *et al.*, 2020; Wang *et al.*, 2021; Junaid, Liu, *et al.*, 2022; Xu *et al.*, 2022; Zhou *et al.*, 2022).

Several studies have found that ARB and ARGs are enriched in the microplastisphere, with concentrations exceeding the surrounding water and other biofilms on surfaces such as glass, ceramics and rocks (Zettler, Mincer and Amaral-Zettler, 2013; Laverty *et al.*, 2020; Zhang *et al.*, 2020). As MPs float or become deposited in the sediment, they enable the accumulation and dispersal of these dangerous hitchhikers, increasing their geographical distribution and posing a threat to aquatic ecosystems and human health (Kirstein *et al.*, 2016; Zadjelovic *et al.*, 2023; Vass, Ramasamy and Andersson, 2024). Furthermore, the biofouling of MPs increases their density, thus facilitating the settlement of MPs in sediments and riverbeds, turning these areas into long-term reservoirs of ARBs and ARGs embedded in the microplastisphere (Wu *et al.*, 2020; Li *et al.*, 2023). These sedimented MPs can be resuspended into the water column, especially following disturbances such as heavy rainfall or flooding which stir up the sediment (Xia *et al.*, 2023). This process results in the redistribution of ARBs and ARGs, further contaminating the aquatic environment and potentially leading to human and animal exposure (Zarean *et al.*, 2025).

The consumption of MPs by fish and filter-feeding aquatic organisms, which are part of the human food chain, poses another significant risk. Fish and other aquatic species can ingest MPs along with the associated biofilms, including ARBs and ARGs (Lu, Zhang, Wu and Luo, 2019; Li *et al.*, 2024). This can introduce resistant bacteria and genes into humans through the food chain, highlighting an indirect pathway for exposure to antibiotic resistance from the microplastisphere (Nguyen *et al.*, 2023).

2.8 DETECTION AND ANALYTICAL METHODS USED IN AQUATIC MICROPLASTIC-BIOFILM STUDIES

This section reviews various methods for identifying and quantifying MPs, analysing biofilms on MPs, and detecting *Vibrio* spp. and ARGs.

2.8.1 Identification and Quantification of Microplastics

Various techniques used in sampling, characterising and analysing MPs from the aquatic environment are discussed in the following sections.

2.8.1.1 Sampling

The first step in identifying and quantifying MPs in the aquatic environment is the collection of representative samples. MPs have been sampled from various aquatic environments including oceans, rivers, coastal areas, estuaries, creeks, lagoons, lakes and wastewater (Table 2-1). Sampling depths ranged from surface water (10- 30 cm below the water surface) (J. Wang *et al.*, 2020; Di Pippo *et al.*, 2022; H. Li *et al.*, 2022) to depths of up to 1.5 m (Sucato *et al.*, 2021) and even sediments (Hu *et al.*, 2021; Liang *et al.*, 2023). Sampling of MPs from aquatic environments usually involves either bulk water sampling, such as filtering surface water using a trawl net (usually 300 µm mesh size) (Frère *et al.*, 2018; Lu, Wu and Wang, 2022) or volume-reduced sampling techniques, such as using a vacuum pump (Zhao *et al.*, 2014), or manually sieving a volume of surface water through a metal sieve to trap microplastic debris (Mughini-Gras *et al.*, 2021; Bae and Yoo, 2022). For example, Carson *et al.* (2013) trawled a 61x16 cm manta trawl with a 3 m net (333 µm mesh) for 1 hour at 2 knots to trap plastic debris from surface water. In comparison, studies using volume-reduced sampling techniques filter varying volumes of water from as low as 100 mL (Dubaish and Liebezeit, 2013) to a maximum of 100 L (Song *et al.*, 2014) of surface water.

2.8.1.2 Physical characterization of microplastics

The most widely used method in the literature for MP analysis was proposed by the National Oceanic and Atmospheric Administration of the USA (Masura *et al.*, 2015). First, MP samples are processed with wet peroxide oxidation (WPO) to digest organic matter. Next, density separation is performed using salts like sodium chloride or zinc chloride to isolate the putative MP particles. These particles are then visualized using stereo microscopy, a fast and cost-effective technique that provides a three-dimensional view of the sample illuminated under white light (Zhu *et al.*, 2024). The MP particles are identified based on their distinct morphology and then counted and classified according to their shape, colour, size or chemical composition (Table 2-1) (Bae and Yoo, 2022; R. Li *et al.*, 2022; Xu *et al.*, 2022; Guruge *et al.*, 2024).

2.8.1.3 Reporting Microplastic Abundance

Studies have used different units to report the abundance of MPs. Some studies reported the total count of MP particles in the collected samples (Kirstein *et al.*, 2016; R. Li *et al.*, 2022), whereas others reported MP abundance as the number of particles/L of surface water

(Xu *et al.*, 2022), particles/m^3 (McCormick *et al.*, 2014; Bae and Yoo, 2022), particles/km^2 of sampled area (Di Pippo *et al.*, 2022), or particle density (kg/km^2) (Carson *et al.*, 2013). Notably, the sampling technique influenced the units used to report the results. For studies that used trawl nets where a substantial volume or area was sampled, the MP abundance was often reported in terms of the sampling area (km^2) or the volume of bulk water sampled (m^3). On the other hand, studies that used manual sampling where smaller amounts of water were filtered, typically, ≤100 L, tended to report the total MP count or MP abundance in particles/L of filtered water or particles/g of sediment analysed (Xu *et al.*, 2022).

2.8.1.4 Chemical Characterization of Microplastics

In addition to the physical characterization of MPs, predominantly ATR-FTIR and Raman spectroscopy have been used to identify the chemical composition of plastic debris isolated from the environment (Zettler, Mincer and Amaral-Zettler, 2013; Amaral-Zettler et al., 2015; Frère et al., 2018; Bae and Yoo, 2022; Liang et al., 2023). Both methods allow for the identification of different types of plastics based on how their molecules interact with light. FTIR measures the absorption of infrared light by a sample, whereas Raman spectroscopy measures the inelastic scattering of light (Käppler *et al.*, 2016). Both techniques produce spectra that serve as a unique chemical fingerprint. The spectral data is then compared against a reference database to identify the functional groups and molecular bonds in the sample (Xu *et al.*, 2019). A limitation of FTIR is that it requires the sample to be thin/transparent enough for infrared light to penetrate it, making it challenging to identify plastics when organic compounds or biofilms are attached to the surface. Raman spectroscopy is more robust for the chemical characterisation of plastics because it is less sensitive to the opacity of the sample and offers higher spatial resolution and detailed molecular information at the surface level, making it more suitable for analysing microplastics (Cabernard *et al.*, 2018; Song *et al.*, 2021). The differences in methods used by researchers result from factors such as availability of resources, study objectives and sampling design. The challenge with these variations in methods is that there is difficulty in adequately comparing results from one study to another because of the differences in their analysis and reporting methods. Nonetheless, each method is useful for studying aquatic plastic debris, and researchers must consider the applicability of each technique based on the available resources and study objectives.

2.8.2 Methods for Analysing Microplastic-Associated Biofilms of *Vibrio* spp.

Despite their potential ecological and public health significance, few studies have focused explicitly on *Vibrio* spp. in MP-associated biofilms. The available studies have focused on specific regions, such as the North and Baltic Seas or Chesapeake Bay. Studies investigating *Vibrio* spp. in the MP biofilm usually use culture or molecular techniques such as PCR and qPCR to detect and quantify their abundance. Firstly, biofilms are carefully removed from MP surfaces under sterile conditions. In their study, Kirstein et al. (2016) applied culture-based techniques, which included enrichment in alkaline peptone water to facilitate *Vibrio* spp. growth, followed by selective culturing on CHROMagar™ *Vibrio* to isolate bacterial colonies and MALDI-TOF MS (Mass Spectrometry) for species-level identification. While culture techniques are widely used for identifying *Vibrio* species in environmental samples, they have several limitations when applied to MP biofilms. The non-culturability of some *Vibrio* species, difficulty in isolating biofilm-associated bacteria, selectivity of culture media, and the time-consuming nature of culture techniques complicate its applicability in MP biofilm studies.

Alternatively, molecular techniques such as *16S rRNA* gene sequencing, polymerase chain reaction (PCR) and quantitative PCR (qPCR) can be used for identifying bacterial communities and quantifying *Vibrio* spp. on MPs. Nucleic acid extraction using methods such as Phenol-chloroform (Martínez-Campos *et al.*, 2022), boiling (Laverty *et al.*, 2020), alkaline lysis (Coons *et al.*, 2021) or commercial DNA extraction kits (Pham, Clark and Li, 2021; Lu, Wu and Wang, 2022; Wu *et al.*, 2022; Xu *et al.*, 2022), is the first step in all molecular assays. The extraction of high-quality nucleic acid in sufficient quantity is critical for the success of molecular assays. One of the major challenges with DNA extraction from environmental samples is the presence of organic or inorganic contaminants and artefacts which may inhibit downstream molecular assays (Felczykowska *et al.*, 2015).

16S rRNA sequencing enables the identification of the microbial community on MPs, which is important to understand *Vibrio* spp. in their larger ecological context. For example, in Kirstein *et al.'s* 2019 study, *16S rRNA* sequencing was useful in identifying potentially pathogenic *Vibrio* species and assessing their role in the microbial assemblage, indicating the health risks presented by MPs as vectors for pathogens (Kirstein *et al.*, 2019).

On the other hand, qPCR is highly efficient for quantifying *Vibrio* abundance and detecting specific pathogenic strains and is therefore necessary for risk assessments. For example, Laverty *et al.* (2020) used qPCR to determine the abundance of total *Vibrio* spp. on MP and further isolated three pathogenic species (*V. parahaemolyticus, V. vulnificus* and *V.*

cholerae) from the MP biofilm. *Vibrio* spp. *16S rRNA* gene and species-specific virulence genes primer targeting the hemolysin/cytolysin gene *vvhA* of *V. vulnificus*, the thermolabile hemolysin gene *tlh* of *V. parahaemolyticus* and the molecular chaperone protein *groEL* for *V. cholerae* were used in the study. Their use of end-point PCR with species-specific primers further confirmed the presence of these pathogens, providing a comprehensive representation of *Vibrio* populations on MPs.

Similarly, Oberbeckmann, et al. (2018) applied qPCR to quantify *Vibrio* abundance on MPs in coastal environments of varying salinity gradients. Their findings showed concentrations of up to 6.95 x 10^4 copies/ng on PS plastics near wastewater treatment plant (WWTP) discharge points. In another example, Frère et al. (2018) utilized real-time PCR (qPCR) with species-specific primers to detect *V. aestuarianus* and *V. splendidus* on MPs sampled from the Bay of Brest, finding *V. splendidus* in 77% of the samples.

Based on the observations from these studies, it is deduced that qPCR can provide valuable insights into the presence of potentially pathogenic strains, even when they are absent from surrounding water, and the method's sensitivity is good in environmental assessments. Although *16S rRNA* sequencing provides comprehensive microbial community profiles, qPCR is preferred for precise quantification and strain identification, making both techniques complementary depending on the research focus. For studies seeking to understand *Vibrio* diversity and dynamics, *16S rRNA* sequencing is essential, whereas qPCR is critical for quantifying and identifying specific pathogens, as seen in the various examples of recent studies.

Although qPCR stands out as the prevalent and effective method for rapid detection of *Vibrio* spp. in MP biofilms, there are certain limitations that must be noted. Conventional qPCR methods poorly distinguish between DNA derived from live bacterial cells, inactivated cells and fragments associated with free DNA (Cangelosi and Meschke, 2014). This lack of differentiation in the source of amplified genetic material may result in the overestimation of *Vibrio* abundance in the aquatic environment and MP biofilm. Viability PCRs including the use of Propidium Monoazide (PMA) treatment have been employed in some studies to enhance the accurate quantification of *Vibrio* spp. in aquatic environments and thus adequate assessment of their environmental and public health risks (Wu, Liang and Kan, 2015; Yang *et al.*, 2023).

2.8.3 Molecular Techniques for *tetB* Gene Detection

Molecular techniques commonly used to investigate ARGs in environmental samples such as water and microplastic biofilms include PCR (Zhang *et al.*, 2020), qPCR (Hu *et al.*, 2021; Wang *et al.*, 2021) and next-generation sequencing (NGS) technologies, including shotgun metagenomics (Guruge *et al.*, 2024) and metatranscriptomics (Wu *et al.*, 2022). PCR provides qualitative insight into the presence or absence of the targeted ARGs. Zhang *et al.* (2020) used PCR to detect the presence of 75 ARGs belonging to seven classes of antibiotics, including tetracyclines, sulphonamides, aminoglycosides, quinolones, chloramphenicol, macrolides and b-lactams. qPCR is the most frequently used method for ARG detection in MP biofilm studies because it enables the quantification of ARG abundance and allows researchers to estimate the risks associated with ARG enrichment in the microplastisphere. Most studies investigating MP-associated ARGs have used relative quantification qPCR, expressing the relative abundance of targeted ARGs as a ratio of the total bacterial abundance (as determined by the *16S rRNA* copies) (Hu *et al.*, 2021; Di Pippo *et al.*, 2022; Kim and Yoo, 2024).

Metagenomics analyses the DNA of a microbial community to understand its composition and potential functions, while metatranscriptomics analyzes the RNA transcripts to reveal which genes are actively being expressed (Terrón-Camero *et al.*, 2022). These techniques have been used to identify resistance determinants, including ARGs, MGEs, biocide/metal resistance genes (BMRGs) and virulence genes. Metagenomic and metatranscriptomic methods involve annotating sequences to reference databases to infer resistance determinants (Q. Li *et al.*, 2022; R. Li *et al.*, 2022; Xu *et al.*, 2022). Guruge *et al.* (2024) identified ARGs, BMRGs and MGEs by annotating sequences from shotgun metagenomic sequencing using the BacMet2 and ProGnomes3 databases and estimated their relative abundance expressed as the contigs per kilobase of gene per million (RPKM).

2.9 KNOWLEDGE GAPS AND FUTURE RESEARCH DIRECTIONS

Significant research has focused on microbial diversity in the plastisphere, but there is a lack of consensus on whether plastic biofilms enrich certain bacteria, including pathogens and ARGs. Some studies suggest higher microbial diversity in microplastic biofilms, compared to surrounding environments (Xue *et al.*, 2020; Sucato *et al.*, 2021; Bae and Yoo, 2022), while others report lower diversity (González-Pleiter *et al.*, 2021; Mughini-Gras *et al.*, 2021). Some other studies have found that several factors including the type of MP polymer (Miao

et al., 2019), the colour of MPs (Wen *et al.*, 2020), the biofilm age (Kirstein *et al.*, 2019) and surrounding environmental conditions such as salinity, dissolved oxygen and nutrients(Oberbeckmann, Kreikemeyer and Labrenz, 2018; Nguyen *et al.*, 2022) affect the biofilm composition. Furthermore, while there are several studies on MP abundance in the marine environment, there is limited data on the occurrence of MP in freshwater systems (Lambert and Wagner, 2018).

Further investigation is needed to clarify the factors driving microbial colonization, including the influence of different plastic types, environmental conditions, and biofilm dynamics on ARG enrichment and HGT (Sathicq *et al.*, 2021). Additionally, research should explore how MP-associated ARB and ARGs are transmitted through ecosystems, their potential to bioaccumulate, and their role in pathogen spread and resistance propagation (Junaid et al., 2022). As advancements in NGS technologies offer new insights, future studies should employ robust NGS techniques such as shotgun metagenomics to elucidate these dynamics of the plastic biofilm (Barros and Seena, 2021).

2.10 IMPLICATIONS FOR STUDY DESIGN AND METHODOLOGY

This thorough review of the literature reveals that aquatic microplastisphere research is a complex and multi-faceted research area that presents many challenges, from sample collection to analysis and interpretation of results. The primary consideration for method selection is the objectives of the study. Imaging and visualisation techniques offer valuable insights into the morphology of the microplastisphere, changes in the biofilm community over time and the impacts of biofilm formation on the structure and degradation of MPs. Imaging techniques have also been successful in the identification of macro-structures such as cyanobacteria, diatoms and dinoflagellates in the microplastisphere (Carson *et al.*, 2013; Zettler, Mincer and Amaral-Zettler, 2013; Di Pippo *et al.*, 2022; Miao *et al.*, 2023). However, these techniques do not provide details on the taxonomic identity of organisms in the microplastisphere such as bacteria, fungi and viruses and their functional roles in the microplastisphere. For risk assessment studies, where researchers are interested in investigating the risks posed by microplastic colonisation by pathogenic species, these species can be directly targeted for isolation through culture. Considering the cost of reagents, equipment and level of training required, culture techniques are less expensive than molecular methods. However, culture is more time-consuming and can take 24–48 h or even longer to culture certain slow-growing bacteria. Additionally, many environmental

microorganisms are non-culturable under standard laboratory conditions or may require highly specific and enriched culture medium, thus complicating the application of culture techniques (Colwell, 2000). Metagenomic sequencing has emerged as the most thorough approach to profiling the microplastisphere, as it provides granular details of the microbial community, functional profiles and resistomes. However, this technique is not easily implemented in low-resource settings. Shotgun metagenomic assays are expensive, and it can be difficult to interpret the results with certainty. Alternatively, targeted sequencing of hypervariable gene regions such as the *16S rRNA, ITS* and *18S/23S rRNA* is a widely used technique that enables taxonomic profiling and may even provide information on the functional roles of the microplastic-biofilm community.

Based on the findings from the review of literature, culture and PCR-based techniques are commonly used in studies that target specific genera and their associated ARGs. However, *Vibrio* spp. are known to enter a VBNC state during adverse environmental conditions during which their genes are still detectable using molecular techniques. Therefore, an accurate estimation of their abundance in the microplastisphere may not be possible using culture techniques. On the other hand, qPCR is more suitable and has been widely used for studying *Vibrio* populations and ARGs associated with the microplastisphere. qPCR enables the accurate detection and quantification of *Vibrio* spp. and *tetB* from MP samples and can be used to compare water and plastic-biofilm samples. However, strict attention must be given to gene target selection and primer design to ensure the success of qPCR assays. Therefore, this study will use molecular techniques such as PCR and qPCR to detect quantify and compare the abundance of *Vibrio* spp. and *tetB* in MP-associated biofilms and surrounding water.

CHAPTER 3: GENERAL METHODOLOGY

3.1 INTRODUCTION

This chapter provides a description of the study area and the general materials and methods. The chapter also highlights the land-use activities influencing the selected study sites.

3.2 STUDY AREA

The Kat River catchment, located in the Amathole District, is surrounded primarily by agricultural activities (Farolfi et al., 2010; Nontongana et al., 2014). Four sampling sites (Figure 3-1) were selected in the Kat River. Two sites (K1 & K2) are located in the middle catchment within Fort Beaufort, an urban area with semi-formal settlements around both sites, and K2 was located downstream of a WWTW discharge point. The other two sites (K3 & K4) were located in the upper catchment, within the town of Balfour and the surrounding land was used for commercial-scale citrus farming.

The Swartkops River flows through the highly urbanised and industrialised Nelson Mandela Bay Municipality (Adams et al., 2019; Mack et al., 2024). Six sampling sites were selected in the Swartkops River. One site (S5) was situated in the Chatty River, a tributary of the Swartkops River, within the estuarine area near Swartkops village. The other five sites were located upstream of the tidal head, in the river's freshwater reaches. S1 was located downstream of a commercial-scale livestock farm and received effluents from a nearby abattoir. S2 was located downstream of a wastewater treatment plant and received effluents from the treatment plant. S3 and S4 were situated in the heavily industrialised town of Uitenhage. S6 was deemed a control site due to its relatively remote location with minimal urban and industrial activity, although large-scale crop farms surrounded it.

The selection of the Kat and Swartkops rivers and the individual sampling points were based on the presence of various anthropogenic influences, including industrial, agricultural, and urban pollution, that contribute to plastic waste and the survival and preponderance of these microorganisms.

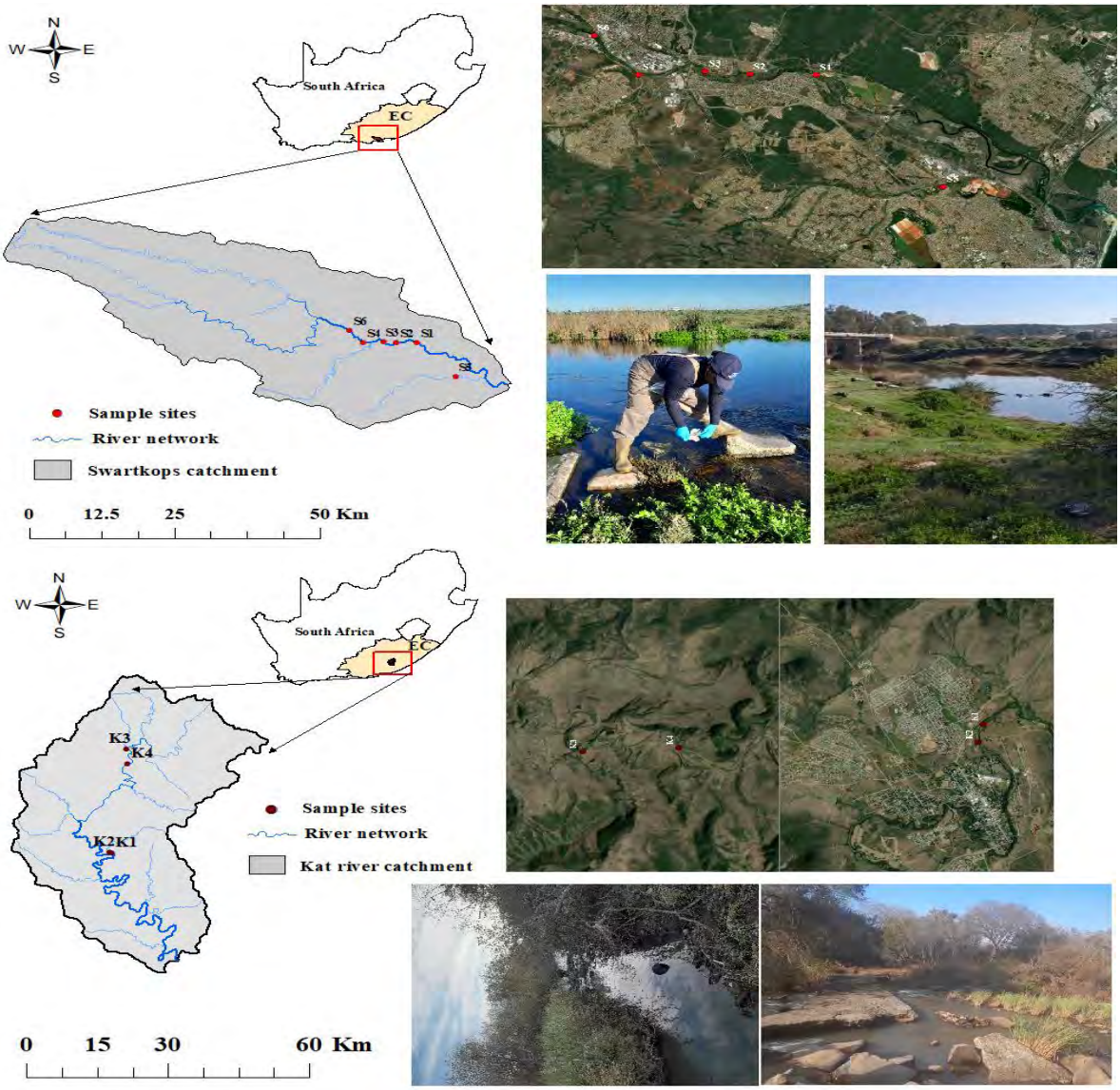

Figure 3-1. Maps of the Swartkops and Kat rivers, Eastern Cape, South Africa. Red dots indicate sampling points. Side panels show a Google Earth satellite view of the sampling sites (top right) and river conditions at some study sites during sampling visits (bottom right). Maps were created using the application ArcMap version 10.3.1.

3.3 SAMPLE COLLECTION

Water and MP samples were collected from ten different sampling sites from the Swartkops and Kat rivers in Eastern Cape, South Africa (Figure 3-1). Samples were collected in March and July 2023. The samples were collected in March (late summer) and July (mid-winter) to capture river dynamics during contrasting climatic conditions. These months were selected as they provided an opportunity to observe potential variations in microbial and environmental parameters which may influence *Vibrio* spp. populations and antibiotic

resistance profiles. During the sampling periods, weather conditions in Swartkops and Amathole showed variations. In March, the Swartkops River area had an average temperature of 21°C, with humidity averaging 78%, while the Kat River area averaged 22°C with 76% humidity. In July, Swartkops averaged 14°C and 75% humidity, while Amathole recorded 16°C and 63% humidity. Pressure ranged from 1001–1027 mbar across regions, reflecting transitional weather patterns during sampling. It is important to note that the intention was not to conduct a seasonal study, as the sampling design focused on maximising site-specific variability and understanding the ecological drivers across a broader temporal and spatial framework. As part of a larger study, this limited temporal snapshot complements other data points to elucidate the environmental factors influencing *Vibrio* spp. and resistance gene dynamics in urban and agricultural river systems in Eastern Cape, South Africa.

The sampling technique used was informed by previous studies (Bae and Yoo, 2024; Mughini-Gras *et al.,* 2021; Song *et al*., 2014). Approximately 2 L of surface water was collected at each of the study sites. MP samples were collected at each site by filtering ~100 L of surface water using a stacked setup of stainless-steel sieve (mesh sizes 5 mm & 250 µM). After filtration, the particles collected on the 250 µM sieve were visually inspected and organic debris, rocks and organisms trapped in the sieve were discarded. Putative MP was rinsed using sterile distilled water and transferred into a sterile 1 L glass bottle. The MP samples collected from each site were divided into two portions. One portion was used to characterise the abundance and types of MP, and the second was used for DNA extraction and assessing the abundance of target genes in the MP biofilm. In total, 56 samples were collected during the study period (20 water and 36 plastic debris samples). Concurrently, temperature (°C), salinity (ppt), dissolved oxygen (mg/L), pH, and turbidity (NTU) of river water were measured at each study site using the Hanna multiparameter water quality meter model H198 during each sampling. The equipment was calibrated before sampling according to the manufacturer's instructions, and the probe was rinsed with distilled water after each site measurement to avoid cross-contamination. In addition, 250 mL of water samples were collected for laboratory analysis of total nitrates (NO_3^-), total nitrites (NO_2^-), total phosphates (PO_4^{3-}) and ammonia (NH_4^+). Nutrient levels of water samples were analysed using a Shimadzu mini 1240 spectrophotometer at 388 nm following the standard protocols of the American Public Health Association (APHA) (Rice et al., 2012) and Velghe and Claeys (1983). A standard curve was developed for each nutrient by measuring the absorbance of standard solutions with known concentrations. Analyses of nutrient

concentrations in each sample were conducted in triplicate to ensure the precision of measurements. Procedural blanks were included in each assay to correct for contamination and ensure instrument accuracy. The average absorbance values from the triplicate measurements were inputted into the standard curve equation to estimate the nutrient concentration for each sample. Nutrient concentrations were expressed as mg/L. These measurements are necessary because environmental factors such as temperature, salinity and nutrients significantly influence the abundance and distribution of *Vibrio* (Sampaio *et al.*, 2022). All the collected water and MP samples were kept on ice in a cooler box and transported immediately to the laboratory for further analysis.

3.4 MICROPLASTICS PROCESSING

The MP processing protocol was adapted from the methods described by Masura et al. (2015). The MP samples were transferred into a 500 mL beaker and oven-dried at 70°C until completely dry. After drying, Wet Peroxide Oxidation (WPO) was conducted to digest the remaining organic matter in the sample. Twenty (20) mL each of 30% Hydrogen peroxide (H_2O_2) and 0.05M Fe (II) solution were added to the beaker. The mixture was boiled at 75°C for a minimum of 30 minutes until all visible signs of organic matter had disappeared. The digested mixture was subjected to density separation using 5M Sodium Chloride (NaCl) to isolate the MP. Settled solids were drained from the beaker and discarded. The floating solids presumed to be MP were filtered and collected using a 0.45 µm glass microfibre filter paper. The filter paper was placed in a covered petri dish and left to air dry overnight before microscopic examination. The MP was visually sorted using a light microscope fitted with a camera. The number and shape of the identifiable MP were recorded. Classification of the shape was based on the description posited by Su et al. (2016); MP with a slender and slightly elongated appearance was classified as fibre, round MPs with spherical shapes as pellets, thin transparent/ translucent particles were classified as films, and irregularly shaped, dense particles were regarded as fragments. The relative abundance of MPs was calculated based on the total MP particle count per site and recorded as the number of particles/L water (Bouwman *et al.*, 2018)

3.5 DNA EXTRACTION

In triplicate, 500 mL of water sample from each site was concentrated by centrifuging at 14,000xg for 20 minutes, and the resulting pellet was collected. The supernatant was then filtered through a 0.2 µm Nylon NS membrane filter (Sartorius Stedim Biotech, Göttingen, Germany) using a vacuum pump, and the residue was pooled together with the pellet for DNA extraction. Genomic DNA was extracted from the plastic biofilm and water samples using the DNEasy PowerSoil Pro Kit (Qiagen, Valencia, CA, USA) according to the protocol recommended by the manufacturer. DNA extractions were conducted in triplicate. The purity and concentration of the extracted DNA were determined via 1% agarose electrophoresis and Nanodrop spectrophotometry. A single compact band on an agarose gel visualised under UV light indicated the successful gDNA extraction. $A_{260/280}$ ratio between 1.8–2.0 and $A_{260/230}$ ratio between 2.0–2.2 were targeted for spectrophotometry, indicating the absence of contaminants such as chaotropic salts, EDTA, non-ionic detergents, proteins and phenol (Boesenberg-Smith, Pessarakli and Wolk, 2012; Viljoen, Booysen and Sreenisvasan Tantuan, 2022). The presence of contaminants can inhibit downstream PCR and qPCR reactions. DNA samples were stored at -20ºC until further analysis.

CHAPTER 4: DEVELOPMENT AND VALIDATION OF A NOVEL PRIMER PAIR TARGETING *RECA* FOR THE DETECTION OF *VIBRIO* SPP. IN ENVIRONMENTAL SAMPLES

Publications based on this chapter:

Nnadozie, H. O. and Nnadozie, C. F. 'Design and validation of a novel primer pair targeting *recA* for the detection of *Vibrio* spp. in environmental water samples.' Under review at *Environmental DNA*

4.1 INTRODUCTION

Rapid, accurate, and efficient tools for the detection and quantification of *Vibrio* in the environment are crucial for understanding their distribution, risk assessment, outbreak investigations and disease surveillance (Yin *et al.*, 2018; Bonny *et al.*, 2022). Most studies that attempt to detect and quantify specific *Vibrio* pathogens or *Vibrio* genera in the environment rely on enrichment methods via selective culture and thereafter apply PCR to identify targeted species. However, *Vibrio* spp. in the aquatic environment may respond to adverse environmental conditions such as changes in salinity and temperature by entering viable but non-culturable (VBNC) states. Isolation of bacteria is impossible in this state because adaptation to *in vitro* conditions for growth is difficult (Huang *et al.*, 2021). However, they still pose a health threat to individuals consuming contaminated water or food in such states, as they maintain virulence factors.

Molecular biological DNA-based diagnostic methods such as polymerase chain reaction (PCR) and real-time PCR (qPCR) are advantageous because they can be used to detect the presence of bacteria directly in the sample, bypassing culture and isolation (Kim *et al.*, 2015). Accurate detection of a bacterial species by PCR relies on the careful selection of a target gene and the design of primers. The chosen target region should be species-specific and ubiquitous among the target population. Most studies employ species-specific virulence genes to detect *Vibrio* pathogens of interest (Loo *et al.*, 2022). For example, the thermostable direct hemolysin gene (*tdh*) and thermostable direct hemolysin-related gene (*trh*) and thermolabile haemolysin gene (*tlh*) are well-documented virulence markers of *V. parahaemolyticus* and have been used for its detection in aquaculture, marine and brackish water (Johnson *et al.*, 2012; Park *et al.*, 2013; Han *et al.*, 2015; Patel *et al.*, 2018; Deng *et*

al., 2020). Similarly, the vulnificus hemolysin gene (*vvh*) is a widely used marker for *V. vulnificus* (Neogi *et al.*, 2010; Dickinson, Lim and Jiang, 2013; Yin *et al.*, 2018; Deng *et al.*, 2020; Diner *et al.*, 2021). The groEL chaperone encoding gene and collagenase gene have also been used as markers for the detection of *V. alginolyticus* in mariculture and environmental water samples (Ahmed *et al.*, 2016; Y. Yu *et al.*, 2022). Cholera toxin enzymatic subunit A (*ctxA*), cytotoxic haemolysin A (*hlyA*) and subunits of the outer membrane pilus protein (ompF, ompW) genes have been targeted for the detection of toxigenic and non-toxigenic *V. cholerae* (Park *et al.*, 2013; Daboul *et al.*, 2020; Anas *et al.*, 2021; Diner *et al.*, 2021; Bhandari *et al.*, 2023). There are, however, pitfalls to this approach because environmental variants of *Vibrio* may not possess the commonly targeted virulence genes, or these genes may not be expressed, limiting the sensitivity of this approach for the detection of potentially pathogenic *Vibrio* species (Loo *et al.*, 2022). For instance, Singh *et al*. (2001), in their evaluation of virulence factors in clinical and environmental strains of *V. cholerae* O1, O139, non-O1, and non-O139 strains, concluded that *V. cholerae* strains, in the absence of cholera toxin, NAG-specific heat-stable toxin, or TCP and OMP, *V. cholerae* O1, O139, and non-O1, non-O139 strains that have clinical or environmental origins, can cause diarrhoea by a mechanism entirely different from that of the toxigenic *V. cholerae* O1 and O139 strains (Singh *et al.*, 2001). The absence of commonly targeted virulence genes in environmental strains suggests that some strains with virulence not captured by the chosen primers might be overlooked. This can have profound impacts for pathogen detection and risk assessment studies.

Alternatively, housekeeping genes have been explored to detect the *Vibrio* genera rather than a single species from environmental samples. Housekeeping genes are more robust than virulence genes since they are ubiquitous and highly conserved among the *Vibrio* genera (Sawabe, Kita-Tsukamoto and Thompson, 2007). The most commonly reported housekeeping gene for the molecular detection and/or quantification of *Vibrio* genera in environmental water samples is the *16S rRNA* gene (Caburlotto *et al.*, 2012; Carvalho *et al.*, 2016; Dubert *et al.*, 2016; Liang *et al.*, 2019; X. Wang *et al.*, 2019; Laverty *et al.*, 2020; Li *et al.*, 2020). Another assay reported in the literature for the detection of *Vibrio* genera in environmental water samples is the primer targeting the RNA polymerase subunit (*rpoA*) developed by Dalmasso *et al.* (2009).

Previous studies have established that the recombinase A (*recA*) gene, a housekeeping gene functioning in recombinational DNA repair, is one of the most highly conserved genes in the *Vibrio* genus (Thompson *et al.*, 2004; Sawabe, Kita-Tsukamoto and Thompson, 2007;

Pascual *et al.*, 2010). Thompson *et al.* (2004) proposed the *recA* gene as an alternative phylogenetic marker for the family *Vibrionaceae* as it was more discriminatory than the *16S rRNA* gene among closely related genera such as *Photobacterium, Gromontia, SaliniVibrio* and *Listonela* (Thompson *et al.*, 2004). Crisaffi *et al.* (2014) studied the stability of *recA, gyrB* and *16S rRNA* gene expression under various stress conditions in *V. anguillarium* isolated from environmental water samples. Their results showed that *recA* was more stable under stress conditions, including salinity, temperature and Fe3+ (Crisafi et al., 2014). Therefore, *recA* is a ubiquitous and stable target suitable for detecting *Vibrio* spp. in the environment.

There is a need to continuously update the molecular tools available for the detection of *Vibrio* spp. and for the development of specific and sensitive primers to detect *Vibrio* spp. directly from environmental samples, bypassing the need for culture and providing a rapid and effective means of estimating the occurrence and abundance of *Vibrio* in the environment.

In this study, novel primers targeting the *recA* gene were designed and tested in-silico and experimentally for the detection of *Vibrio* spp. The performance of the designed primers, ergo their specificity, sensitivity and amplification efficiency, were comparatively assessed with a widely used *16S rRNA* primer pair (V.16S-700F/1325R) to detect *Vibrio* spp. directly from river water and microplastic biofilm samples.

Beyond the practical value offered by the development of culture-independent molecular tools to address current challenges in environmental monitoring and microbial ecology, this aspect of the study provides a comprehensive insight into the presence or absence of *Vibrio*, specifically in river water samples. The findings underscore the significance of understanding the presence or absence of these genes in river water, revealing insights into potential reservoirs and risks of disease transmission from the river environment.

4.2 METHODOLOGY

4.2.1 Primer design and In-silico primer specificity validation

Reference sequences for the *recA* gene of 15 *Vibrio* spp. were retrieved from the NCBI gene database (https://www.ncbi.nlm.nih.gov/gene). The list of species and Gene ID numbers are given in Table 4-1. The sequences were aligned using the ClustalW algorithm via MEGA (version 11) and primers were designed using PrimerDesign M (https://www.hiv.lanl.gov/content/sequence/PRIMER_DESIGN/primer_design.html). The

PrimerDesign M tool was selected based on its capability to design primers for highly variable sets of aligned sequences (Brodin *et al.*, 2013; Yoon and Leitner, 2014).

The primers were designed to target variable and conserved regions of the *recA* gene across the *Vibrio* genus (Figure 4-1). Various probabilities were obtained for forward and reverse primers. The following criteria were used to select the best primer pair for in-silico and laboratory evaluation: 1) primer pair with low complexity, 2) low self-complementarity and low complementarity in the 3' end (i.e., ≤ 6), 3) amplicon length between 100–1000bp, 4) GC content ≤ 60%, 5) melting temperature (Tm) between 55 - 70 °C and 6) positive amplification of various *Vibrio* spp. via in-silico amplification (Bustin, Mueller and Nolan, 2020). In-silico specificity of the primers was evaluated using NCBI-BLAST against non-redundant nucleotide data in GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi). In-silico PCR amplification was conducted using online software http://In silico.ehu.es/ against 25 *Vibrio* strains listed in Table 4-2 (Bikandi *et al.*, 2004; San Millán *et al.*, 2013). Finally, the selected primers were synthesised by Inqaba Biotechnical Industries Ltd., South Africa, as follows: 5' – GGTCAAATTGAAAAGCAATT -3' (forward) and 5' – ACTTCDTCRCCTTCTTTGAT -3' (reverse).

Table 4-1. *Vibrio* spp. *recA* gene reference sequences used for primer design

Species	Gene ID	Reference assembly code
V. tarriae	88784085	NZ QKKK01000013.1
V. rotiferanus	47655748	NZ CP018312.1
V. parahaemolyticus	1190074	NC 004603.1
V. owensii	47100345	NZ CP019959.1
V. mimicus	93953449	NZ UHIG01000001.1
V. jasicida	48230025	NZ CP025792.1
V. harveyi	83580638	NZ CP125875.1
V. fluvialis	29384735	NZ CP014035.2
V. diazotrophicus	4025721	NZ CP151842.1
V. diabolicus	57839701	NZ CP042451.1
V. cholerae	69720701	NZ CP043554.1
V. campbellii	67376278	NZ CP026321.1
V. antiquarius	45026742	NC 013456.1
V. alginolyticus	75166657	NZ CP098034.1
V. vulnificus	93895847	NZ CP012881.1

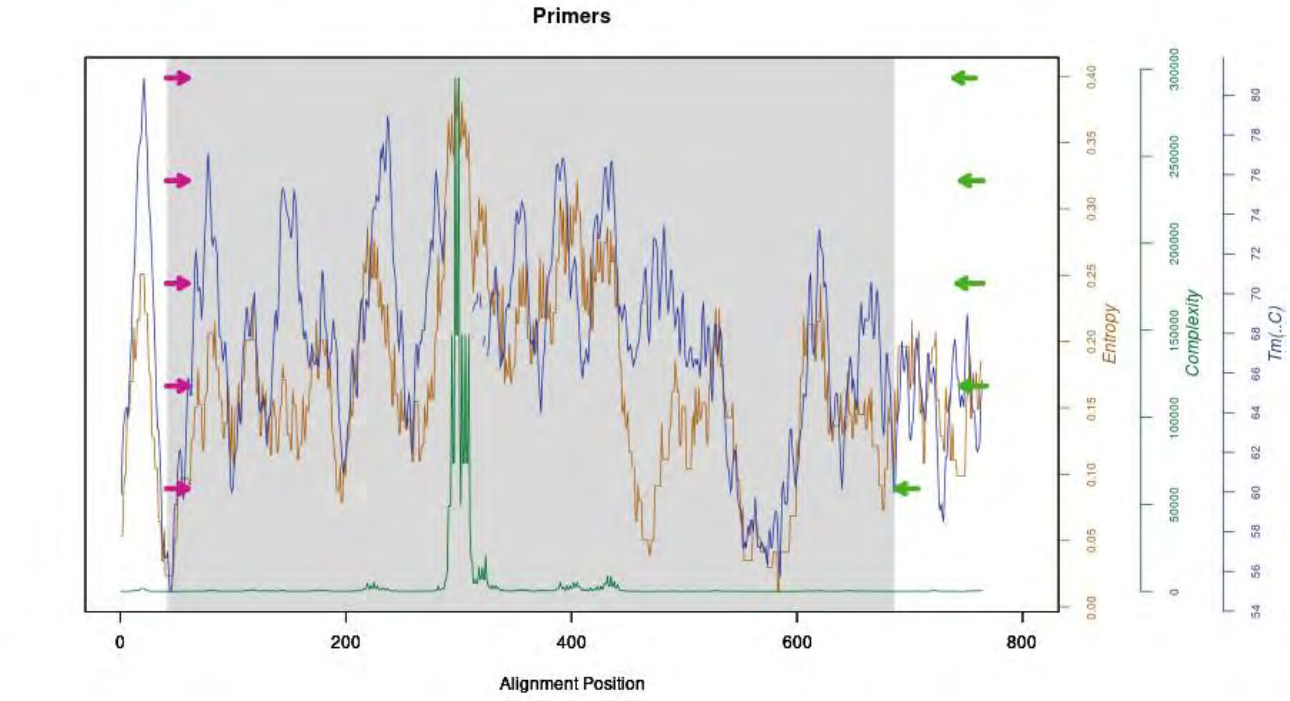

Figure 4-1. PrimerDesign M output summary for multiple fragment options from the *Vibrio* spp. *recA* gene sequence. Gray region: region of interest, pink arrows: 5' primers, green arrows: 3' primers.

Table 4-2. List of *Vibrio* strains tested during In-silico PCR amplification

***Vibrio* Strains**
V. alginolyticus NBRC 15630 = ATCC 17749
V. cholerae LMA3984-4
V. campbellii ATCC BAA-1116
V. cholerae MJ-1236
V. cholerae
V. cholerae IEC224
V. cholerae O395 chromosome 1
V. cholerae M66-2
V. anguillarum 775
V. fischeri MJ11
V. cholerae O395
V. cholerae O1 str. 2010EL-1786
V. fischeri ES114

V. parahaemolyticus BB22OP
V. furnissii NCTC 11218
Vibrio sp. EJY3
V. parahaemolyticus O1:Kuk str. FDA_R31
V. parahaemolyticus O1:K33 str. CDC_K4557
V. harveyi ATCC BAA-1116
V. parahaemolyticus RIMD 2210633
V. vulnificus YJ016
Vibrio sp. Ex25
V. vulnificus MO6-24/O
V. vulnificus CMCP6
V. splendidus LGP32

4.2.2 Bacterial strains

Seven certified strains of South African marine *Vibrio* species were obtained from the Institute for Microbial Biotechnology and Metagenomics (IMBM), South Africa, and one strain of *Escherichia coli* was obtained from the Department of Microbiology, Biochemistry and Bioinformatics, Rhodes University, South Africa (Table 4-3). The halophilic *Vibrio* strains were grown on Marine agar prepared according to Zobell (1941), while *E. coli* was grown in Tryptic soy Agar (Sigma-Aldrich, Germany). The cultures were incubated at room temperature for 24 h. DNA was extracted from each culture using DNeasy Blood & Tissue (Qiagen, Hilden, Germany), according to the manufacturer's protocol. The purity and concentration of the extracted DNA were determined via 1% agarose electrophoresis and Nanodrop spectrophotometry. DNA samples were stored at -20ºC until further analysis.

Table 4-3. Certified bacterial strains used for primers' specificity testing

Species	Source	Strain
V. pomeroyi	IMBM	PE14_21
V. pomeroyi	IMBM	PE14_30
V. gallaecicus	IMBM	PE14_9
V. lentus	IMBM	PE04_57
PseudoVibrio ascidiaceicola	IMBM	PE05_102

PseudoVibrio ascidiaceicola	IMBM	PE12_106
AliiVibrio sifiae	IMBM	PE14_41
Escherichia coli	RU	-

IMBM – Institute for Microbial Biotechnology and Metagenomics, *RU* – Department of Microbiology, Biochemistry and Bioinformatics, Rhodes University

4.2.3 Specificity testing

The *Vibrio* genus *16S rRNA* gene was amplified using the published primer pair V.16S-700F/1325R with the sequence: 5' – CGGTGAAATGCGTAGAGAT – 3' (forward) and 5' – TACTAGCGATTCCGAGTTC-3' (reverse) (Tarr *et al.*, 2007). Subsequently, inter and intra-species specificity of both the developed *recA* and *16S rRNA* primer pairs were assessed by analysing DNA extracted from all the certified *Vibrio* strains and *E. coli*. Due to their shared evolutionary lineage and high sequence similarity in conserved genes among the family Vibrionaceae, cross-amplification between closely related genera might be expected. For example, the *16S rRNA* gene often exhibits >97% similarity between genera in the family Vibrionaceae (Sawabe, Kita-Tsukamoto and Thompson, 2007; Thompson *et al.*, 2009). The *recA* gene on the other hand, offers greater resolution. However, due to the presence of highly conserved regions across genera, cross-amplification may still occur (Klemetsen, Karlsen and Willassen, 2021). The inclusion of closely related strains such as *A. sifiae* was used to determine the discriminatory capacity of the designed *recA* primer. Simplex PCR was carried out on a Veriti™ 96-well thermal cycler (Applied Biosystems, Foster City, CA, USA) with a reaction volume of 50 µL containing 25 µL KAPA Taq Ready Mix (Kapa Biosystems, Wilmington, MA, USA), and 2.5 µL each of forward and reverse primers (0.5 µM), 18 µL ddH_2O, and 2 µL of genomic DNA. Optimisation of PCR conditions for each primer pair was done individually using gradient cycles. The optimised thermocycling conditions for both the *recA* and *16S rRNA* primers consisted of 5 min at 95ºC, followed by 35 cycles at 95ºC for 2 min, 60ºC for 1 min, 72ºC for a further 1 min and a final extension step at 72ºC for 7 min. Amplicons were resolved by electrophoresis on a 1% agarose gel (CSL-AG100, Cleaver Scientific Ltd. Warwickshire, UK), run in Tris-Borate-ethylenediaminetetraacetic acid (TBE) buffer stained with ethidium bromide (1 mg/mL) for 40 min at 100V, and amplification patterns were visualized.

4.2.3.1 Sequencing and Phylogenetic Analysis

Phylogenetic analysis was conducted to confirm the taxonomic identity and sequence similarity of the pure cultures. The *recA* amplicons were purified using Exo-Sap treatment and sequenced by the Aquatic Genomics Research Platform (AGRP) at the South African Institute for Aquatic Biodiversity (SAIAB). The resultant sequences were visualised and analysed using MEGA version 11. Sequences were trimmed to remove poor-quality reads, and degenerate bases were edited by reviewing the electropherogram to identify the most likely nucleotide. Homology search was performed using the BLASTN sequence similarity search at the NCBI database (Altschul *et al.*, 1990). The ClustalW algorithm in the MEGA 11 program was used to conduct multiple nucleotide sequence alignments, including the *recA* sequence fragment of the pure culture isolates, with other sequences acquired from the NCBI database. A phylogenetic tree was constructed in MEGA 11 using the maximum-likelihood approach, the Tamura 3-parameter model, a discrete Gamma distribution and a rate difference of 5 categories. The bootstrap confidence intervals for each node were calculated over 1000 replicates. Sequence from *Campylobacter fetus* CFF00A031 (Accession number NZ_CP059443.1:c185011-183974) was used as an outgroup.

4.2.4 Measurement of primer sensitivity

PCR amplicons of the *16S rRNA* and *recA* gene fragment from *V. pomeroyi* (PE14_30) were purified using the NucleoSpin® Gel and PCR Clean-up kit (Machery-Nagel, Germany). The gene copies in the purified DNA were calculated using the formula

$$Copy\ number = \frac{amount\ of\ amplicon(ng) * 6.0221 \times 10^{23} molecules/mol}{(length\ of\ dsDNA\ amplicon * 660g/mol) * 1 \times 10^{9}\ ng/g}$$

where 6.0221 x 10^{23} molecules/mol is Avogadro's constant, 660 g/mol is the average mass of 1 bp of dsDNA and 1 x 10^{9} is the conversion factor (Prediger, 2024). The gene copies in the purified DNA were calculated to be 2.24 x 10^{10} copies/µL and 8.67 x 10^{10} copies/µL, for *recA* and the *16S rRNA* amplicons, respectively. Subsequently, the amplicons were 10-fold serially diluted in sterile ddH_2O up to 1 copy/µL. The limit of detection (LOD) was used as a proxy to determine the primer sensitivity. PCR was conducted using the optimised conditions previously described, for both the *recA* and the *16S rRNA* primers, with the amplicon concentrations from 10^{9} to 1 copy/µL. Samples were run in three technical replicates along with non-template controls in three different PCR runs to further confirm the repeatability and reproducibility of the amplification. Sensitivity was determined as the minimum

detectable concentration positively amplified in all three PCR runs. At least 8/9 replicates at a particular concentration had to give a positive result to be regarded as detectable.

4.2.5 Amplification efficiency testing

The amplification efficiency of both the *recA* and *16S rRNA* primers was measured to determine whether the primers were suitable for use in quantitative assays such as absolute or relative quantification of the abundance of *Vibrio*. Standards with concentrations ranging from 10^9 to 1 copies/µL were generated from PCR amplicons of *V. pomeroyi* (PE14_30) *recA* gene fragment, and *16S rRNA* gene fragment. qPCR was performed using the Applied Biosystems QuantStudio™ 3 System (ThermoFisher Scientific, Lenexa KS, USA). The same qPCR conditions were used for the two primers. The total reaction volume was 10 µL, consisting of 5.0 µL TB Green® Premix Ex Taq II and 0.2 µL ROX Reference Dye II (Takara Bio Inc., Kusatsu, Shiga, Japan), 0.5 µL each of the forward and reverse primers (0.5 µM), 1.8 µL ddH_2O and 2µL DNA template. A three-step qPCR reaction was performed with the following conditions: 95ºC for 30 s, followed by 40 cycles of 95ºC for 15 s, 60ºC for 30 s and 72ºC for 60 s. Fluorescent signals were captured in the elongation step. The specificity of the reaction for each primer was verified by an evaluation of the melt curves of the qPCR products, which were obtained after the completion of the qPCR cycles using an additional thermal step (95ºC for 15 s, 60ºC for 60 s, and 95ºC for 1 s). Each standard concentration and non-template reaction control (NTC) was run in triplicate for both the *recA* and *16S rRNA* primers. The efficiency of each primer was calculated with the equation E = (10^-1/slope - 1) *100%. The common logarithm of the gene copy number was plotted against the average C_q value of the three replicates for each concentration. The coefficient of regression (R^2) for both primers had to be > 0.99, with a slope between -3.6 and -3.3, corresponding to efficiency of between 90–110%, indicating good suitability for qPCR assays.

4.2.6 Detection of *Vibrio* from water and microplastic biofilm samples

Surface water and MP biofilm samples collected from 6 sites in the Swartkops River and 4 sites in the Kat River (Figure 3-1) were used for validation of the primers' performance. The detection of *Vibrio* from water and microplastic biofilm samples was conducted using both the *recA* and *16S rRNA* primers to allow for a comparative analysis of their performance. The 20 µL PCR reactions were composed of 10 µL KAPA Taq Ready Mix (Kapa Biosystems,

Wilmington, MA, USA), 1 µL each forward and reverse primers (0.5 µM), 6 µL ddH_2O and 2 µL of genomic DNA. PCR reactions were performed using a Veriti™ 96-well thermal cycler (Applied Biosystems, Foster City, CA, USA). The thermocycling conditions described in Section 4.2.3 were used. Amplified products for both primers were examined by electrophoresis on 1% agarose gel (CSL-AG100, Cleaver Scientific Ltd. Warwickshire, UK) stained with ethidium bromide (1 mg/mL). After this, the gels were visualised using a UV imaging system (molecular imager ChemiDocTM XRS+, BIO-RAD).

4.3 RESULTS

4.3.1 In-silico evaluation by BLAST and in-silico PCR amplification

The designed primers showed strong specificity, matching various *Vibrio* spp. sequences in GenBank. The forward and reverse primers corresponded to 79 and 77 *Vibrio* spp. respectively and showed zero to two mismatches with the primer binding sites of all *Vibrio* via BLAST search. The site variations of primer binding regions in various *Vibrio* spp. are given in Appendix 2. In-silico PCR amplification (http://In silico.ehu.es/) produced positive amplification with a 668 bp band, with 17 of the tested strains: *V. cholerae* (8 strains), *V. alginolyticus* (ATCC 17749), *V. parahaemolyticus* (4 strains), *V. harveyi* (ATCC BAA-1116), *V. campbellii* (ATCC BAA-1116), *Vibrio* sp. *Ex25* and *Vibrio* sp. *EJY3*. A screenshot image of the results from in-silico PCR amplification conducted via the online tool at http://In silico.ehu.es/ is presented in Figure 4-2.

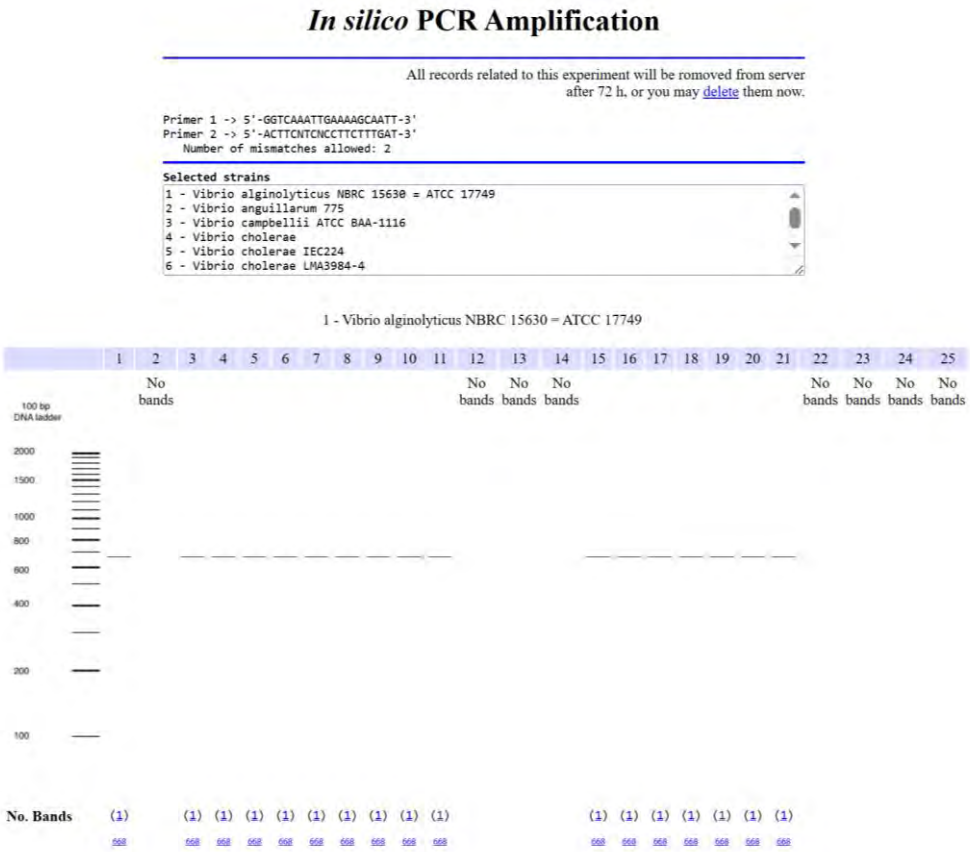

Figure 4-2. In-silico PCR amplification conducted via the online tool at http://In silico.ehu.es/. The designed *recA* primer was tested against 25 *Vibrio* strains (listed in Table 4-2). Positive amplification was detected in Lanes 1, 3-11 and 15-21 with a predicted 668bp product.

4.3.2 Bacterial strains and DNA extraction

Agarose gel electrophoresis of DNA extracted from all the pure culture bacterial strains confirmed the extraction of high molecular weight DNA. A single compact band indicated successful gDNA extraction from all the cultures (Figure 4-3). All samples showed $A_{260/280}$ absorbance ratio between 1.7–1.8, and $A_{260/230}$ absorbance ratio between 1.9–2.2, indicating high-quality DNA yields.

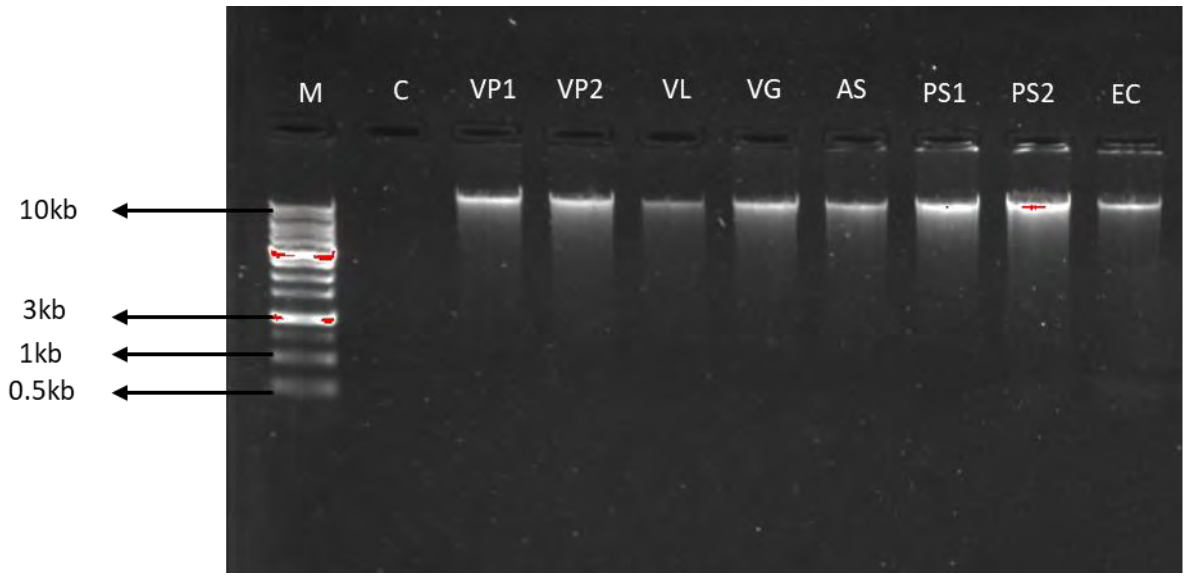

Figure 4-3. A 1% agarose gel showing successful extraction of gDNA from pure culture bacterial strains. M – 1kb DNA Ladder (New England Biolabs®, USA), C - No template/negative control, VP1 – *V. pomeroyi* (PE14_21), VP2 – *V. pomeroyi* (PE14_30), VL – *V. lentus* (PE04_57), VG – *V. gallaecicus* (PE14_9), AS – *A. sifiae* (PE14_41), PS1 – *P. ascidiaceicola* (PE05_102), PS2 – *P. ascidiaceicola* (PE12_106), EC – *E. coli.*

4.3.3 Primer specificity and sequence analysis

For both the *Vibrio 16S rRNA* and the developed *recA* primer, unique amplification bands with the expected amplicon size were observed in PCR reactions with DNA from *V. pomeroyi* (PE14_21 and PE14_30), *V. gallaecicus* (PE14_9), *V. lentus* (PE04_57) and *A. sifiae* (PE14_41). No amplification was observed in the PCR reactions for *E. coli* and *P. ascidiaceicola* (PE05_102 and PE12_106), highlighting the specificity of both primers (Figure 4-4 a, b).

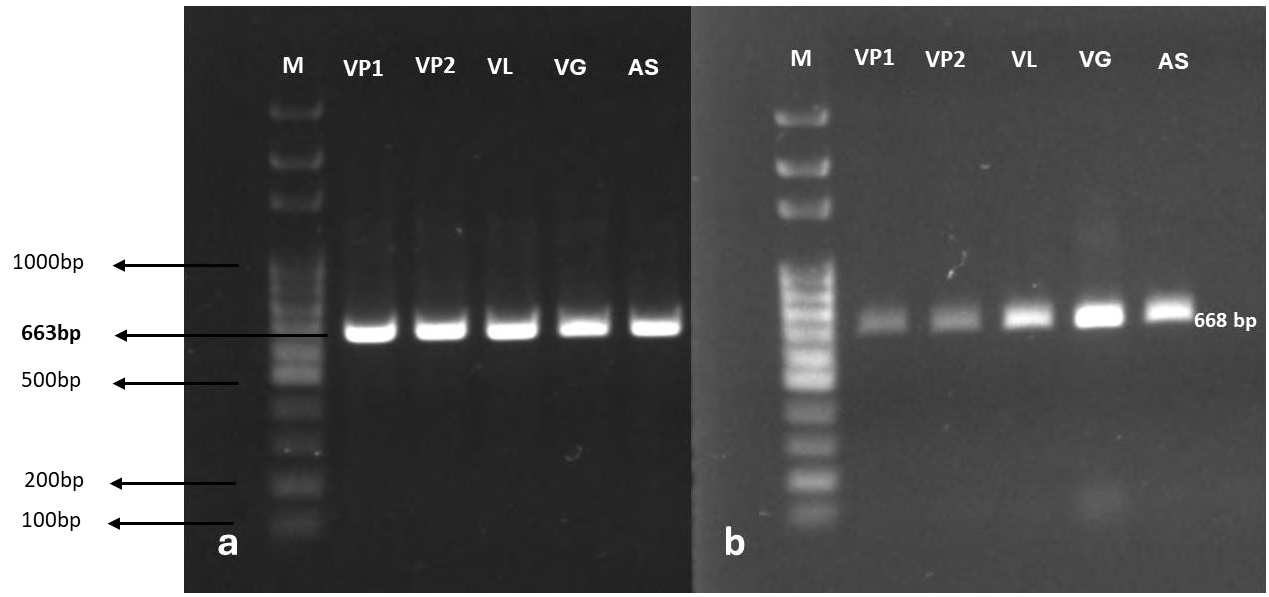

Figure 4-4. Evaluation of the specificity of the a) - the *16S rRNA* gene and b) – designed *recA* primer, using as template DNA from *Vibrio* species and species phylogenetically related to the *Vibrio* genus M – 100 bp DNA Ladder (Solis BioDyne, Estonia), VP1 – *V. pomeroyi* (PE14_21), VP2 – *V. pomeroyi* (PE14_30), VL – *V. lentus* (PE04_57), VG – *V. gallaecicus* (PE14_9), AS – *A. sifiae* (PE14_41).

Based on BLAST searches using the sequenced *recA* fragments, the closest named species match to *V. pomeroyi* (PE14_30) were *V. pomeroyi* strain YSX02 *and V. gigantis* strain SI_2 (510/523 bp match, 98% similarity), *V. pomeroyi* (PE14_21) similarities were *V. pomeroyi* LMG 20537 and *V. gigantis* strain SI_2 (511/519 bp match, 98% similarity), *V. lentus* (PE14_57) similarity was *V. lentus* strain R-3895 (513/520 bp match, 99% similarity), *V. gallaecicus* was *V. gallaecicus* strain VB 5.12 (530/539 bp match, 98% similarity) and *A. sifiae* (PE14_9) similarity was *A. logei* isolate 622f83115ee5131b54a8b4d (562/570 bp match, 99% similarity). The *Vibrio* species included in the phylogenetic analysis clustered with similar species (Figure 4-5), consistent with the results based on the sequencing of other loci (Beaz-Hidalgo *et al.*, 2009).

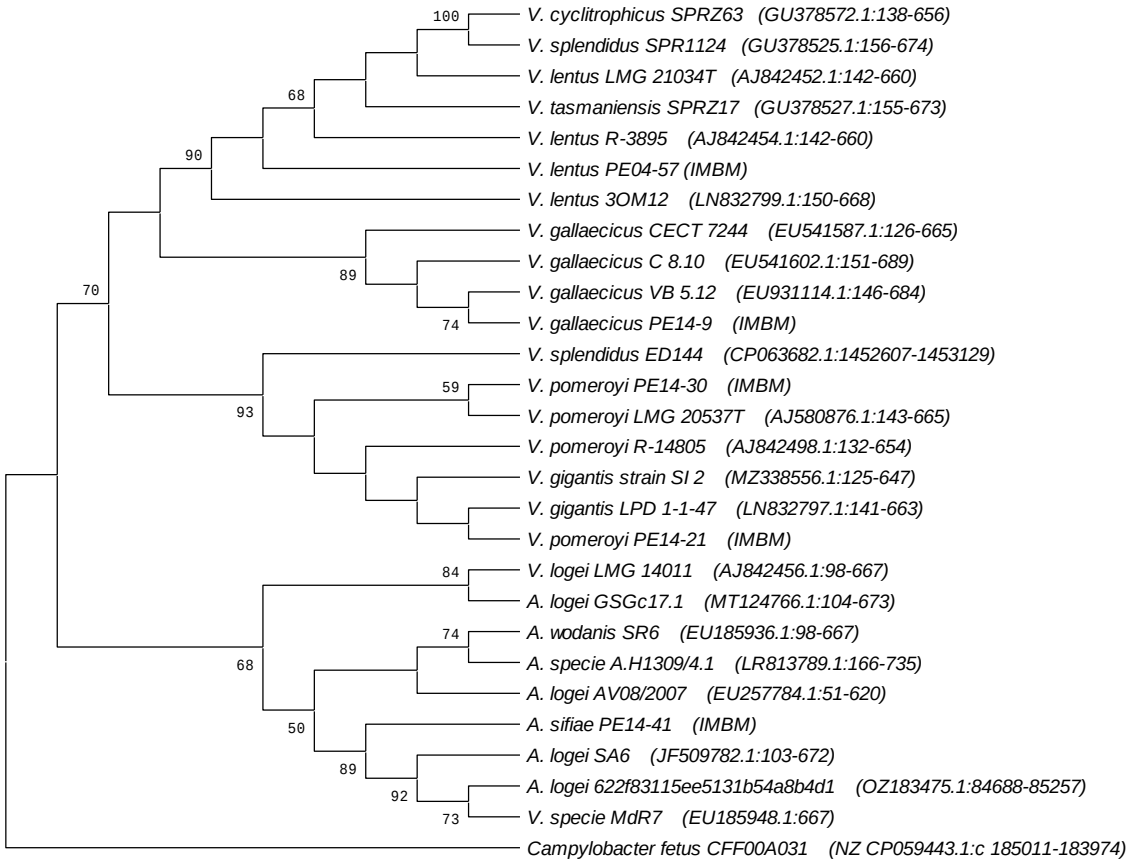

Figure 4-5. Phylogenetic tree based on the Maximum Likelihood method and Tamura 3-parameter model, bootstrapped 1000 times, using *recA* gene sequences of *V. pomeroyi* (PE14_21 and PE14_30), *V. lentus* (PE04_57), *V. gallaecicus* (PE14_9) and *A. sifiae* (PE14_41) obtained from IMBM, as well as other *Vibrio* spp. and *AliiVibrio* spp. sequences downloaded from NCBI GenBank. *Campylobacter fetus* CFF00A031 was used as an outgroup. GenBank sequence accession numbers are given in parentheses. Numbers at the nodes show the percentage bootstrap values. Bootstrap values below 50 are not displayed.

4.3.4 Primer sensitivity

The LOD for the *recA* primer was 10^4 copies/µl, whereas the sensitivity of the *16S rRNA* primer was one order of magnitude greater, 10^3 copies/µL. The *recA* primer showed positive amplification in 9/9 replicates of concentrations between 10^9 to 10^4 copies/µL. However, only 3/9 reactions showed positive amplification at a concentration of 10^3 copies/µL. On the other hand, the *16S rRNA* primer showed positive amplification in 9/9 replicates of concentrations, ranging from 10^9 to 10^3 copies/µL. Neither primer showed any positive amplification for in reactions with template concentrations between 10^2 to 1 copy/µL. Agarose gel amplification

of PCR products at concentrations ranging from 10^9 to 10^3 for both primers are presented in Figure 4-6 (a, b).

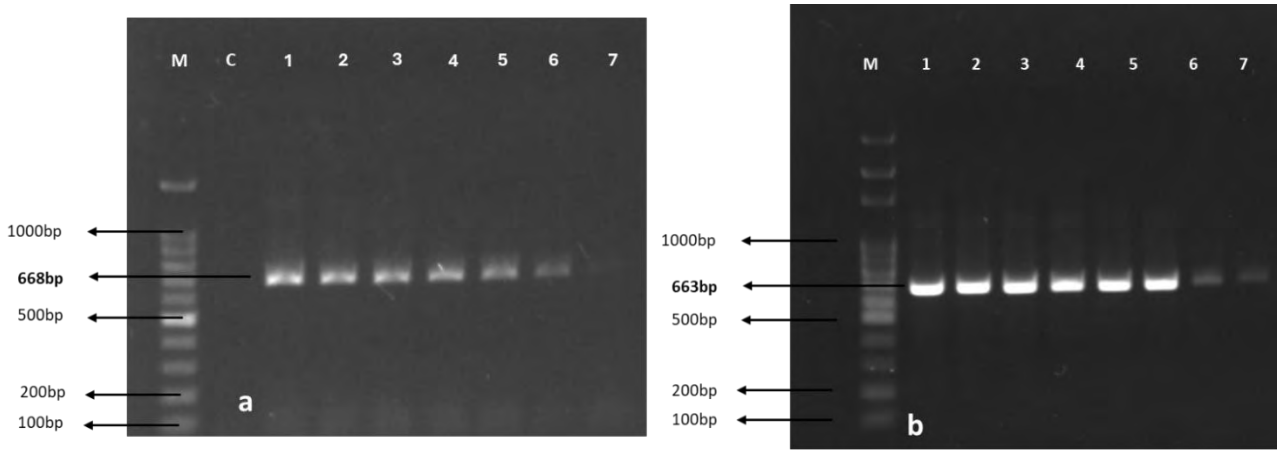

Figure 4-6. Evaluation of primer sensitivity a) – designed *recA* primer, b – *16S rRNA* primer. M – 100bp DNA Ladder (Accuris™ Smartcheck™, USA), C – NTC, Lane 1 – 10^9 gene copies/µL, Lane 2 – 10^8 gene copies/µL, Lane 3 – 10^7 gene copies/µL, Lane 4 - 10^6 gene copies/µL, Lane 5 – 10^5 gene copies/µL, Lane 6 – 10^4 gene copies/µL, Lane 7 - 10^3 gene copies/µL.

4.3.5 Amplification efficiency

For both primers, fluorescent signals remained undetermined via qPCR in the reactions with a concentration of 10^9 gene copies, potentially due to the high concentration of the template DNA which exceeded the equipment sensitivity, indicating the upper limit of quantification (LOQ) for the Applied Biosystems QuantStudio™ 3 System (ThermoFisher Scientific, Lenexa KS, USA). Both primers showed good amplification efficiency, with R^2 values > 0.99. For the *recA* primer, a plot of the C_q values versus the logarithm of the gene copy number resulted in a slope of -3.48, corresponding to an efficiency of 94% (Figure 4-7 a), while the *16S rRNA* primer showed a slope of -3.75, corresponding to an efficiency of 85% (Figure 4-7 b). Melt curve analysis for both primers showed a single peak - 85.44 ± 0.22 °C for *recA* and 88.16 ± 0.15 °C for the *16S rRNA* primer (Figure 4-8 a, b).

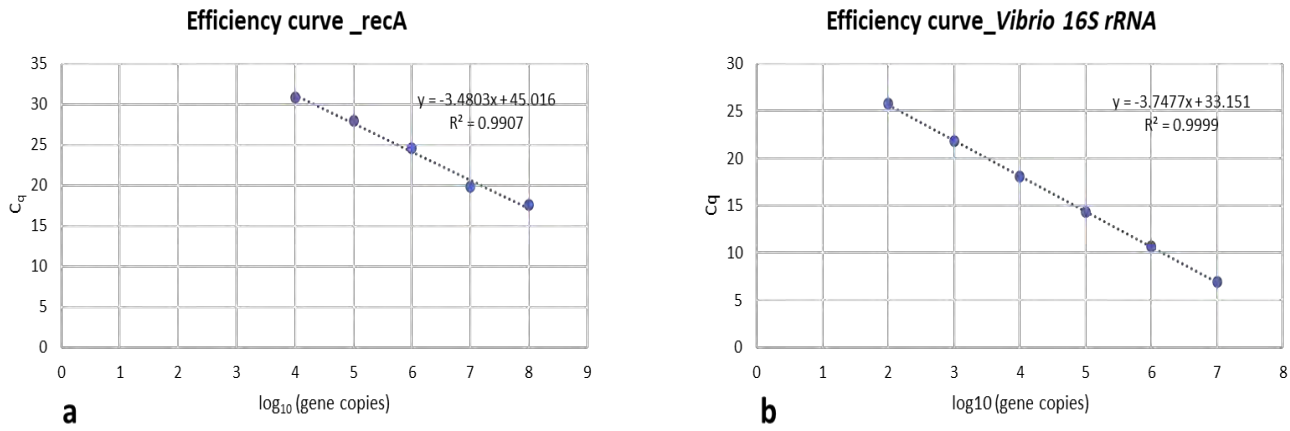

Figure 4-7. Amplification efficiency curve for a) the designed *recA* primer with concentrations ranging from 10^8 to 10^4 copies/µL and b) the *Vibrio 16S rRNA* primer with concentrations ranging from 10^7 to 10^2 copies/µL. The $\log_{10}$ gene copy number is plotted against the average C_q value of the three replicates for each concentration. The standard curve equation and R^2 values are also displayed in the plot.

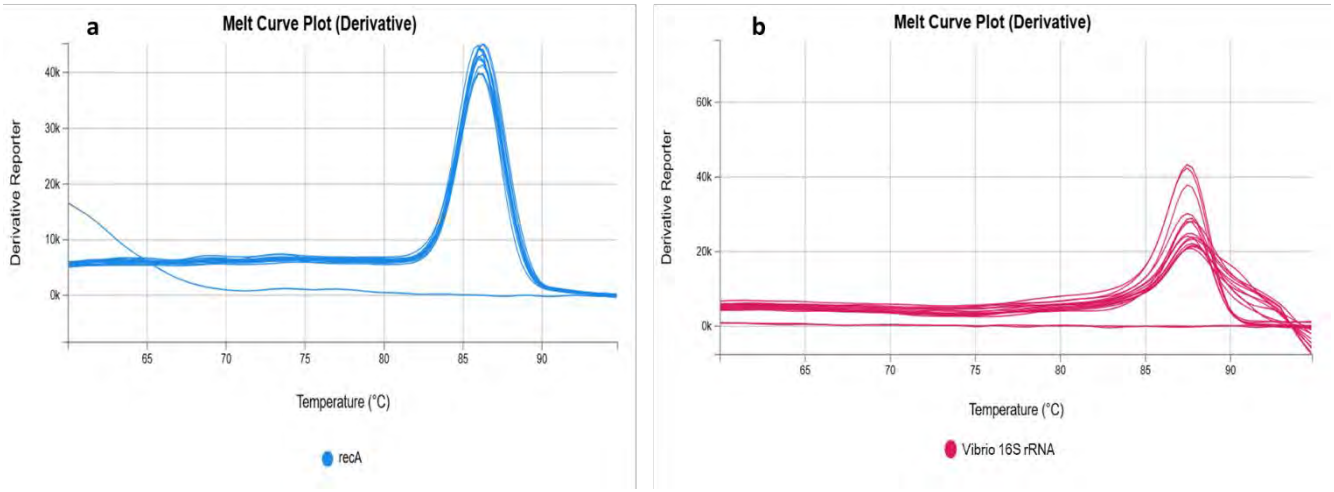

Figure 4-8. Melt curve plot for a) - the designed *recA* primer and b) - the *Vibrio 16S rRNA* primer. The temperature is plotted against the negative derivative of the fluorescence divided by the derivative of the temperature. The peaks around 86ºC (panel a) and 88ºC (panel b) indicate positive amplification of the template DNA. No amplification was observed in the NTC.

4.3.6 PCR detection of *Vibrio* spp. from water and microplastic biofilm samples

Both primers were able to detect *Vibrio* spp. from water and microplastic biofilm samples, however, the *Vibrio 16S rRNA* primer showed a higher detection rate (Figure 4-9). End-point PCR yielded positive amplification in 19/20 (95%) water samples and 15/36 (42%) plastic

biofilm samples using the designed *recA* primer, whereas *Vibrio* spp. was detected in 100% of the water and 100% of the plastic biofilm samples using the *Vibrio 16S rRNA* primer. The higher detection rate using the *16S rRNA* primer can be attributed to its higher sensitivity. Instances of positive amplification of the *recA* gene and *16S rRNA* gene from water and microplastic biofilm samples are shown in Figures 4-10 and 4-11 respectively.

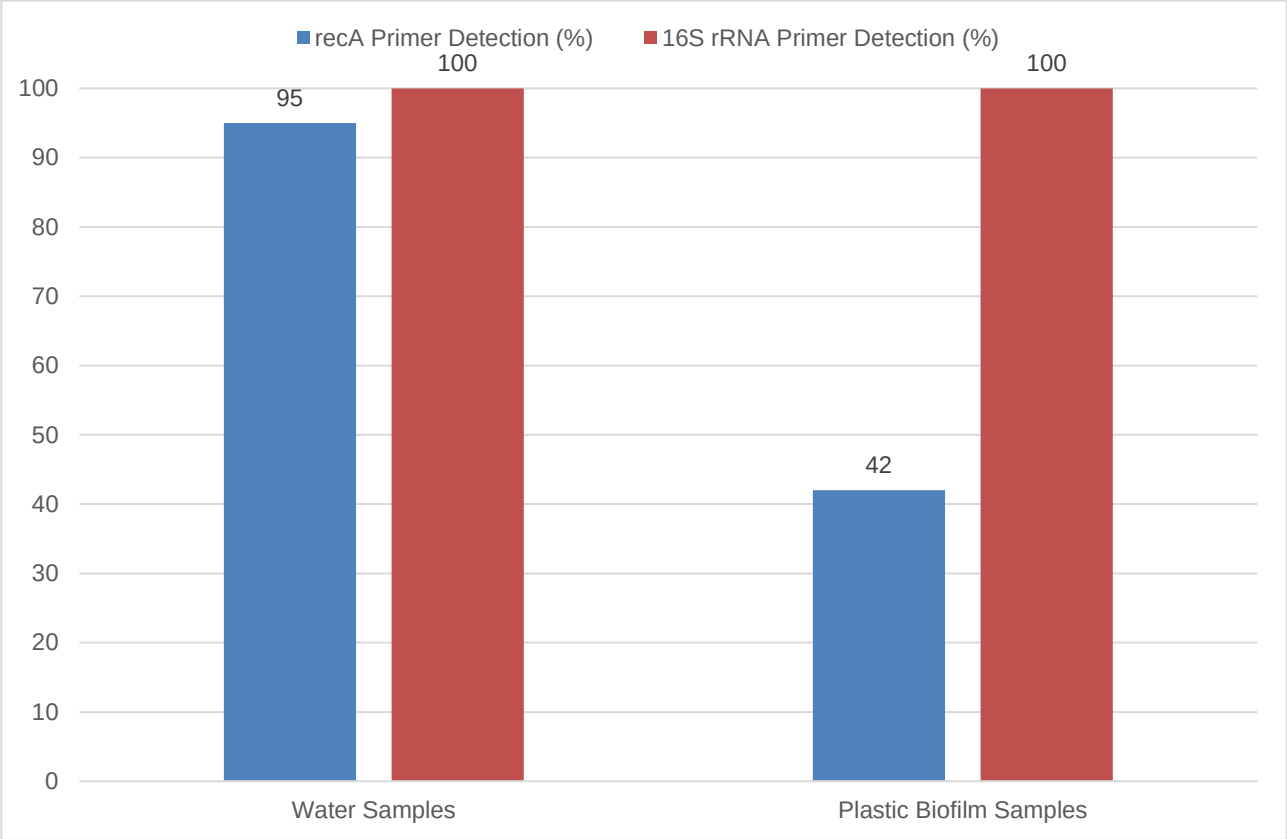

Figure 4-9. Comparison of *Vibrio* detection rates in environmental samples using *recA* as opposed to *16S rRNA* Primers.

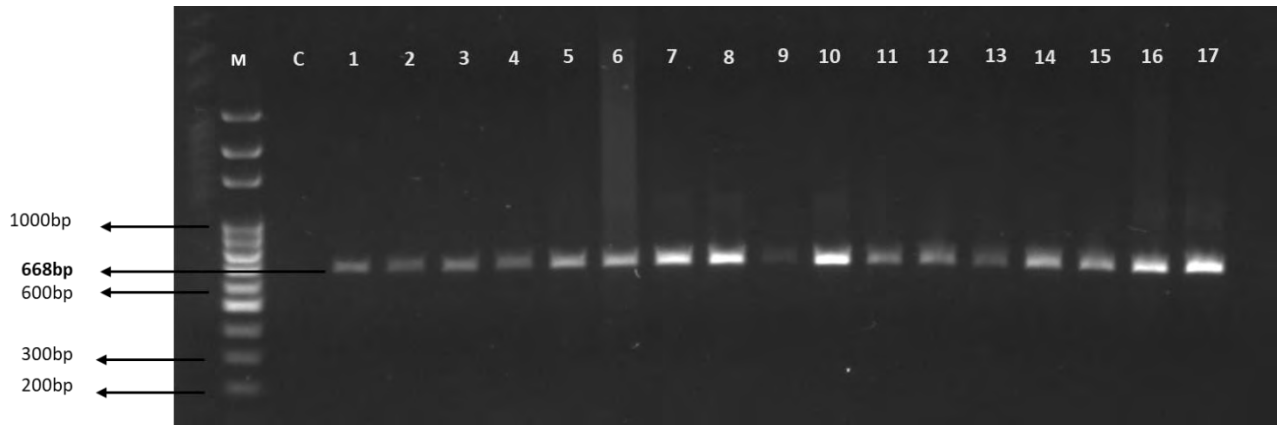

Figure 4-10. A 1% ethidium bromide-stained agarose gel showing an instance of the detection of *Vibrio* spp. from water and microplastic biofilm samples using the designed *recA* primer. M - 100bp DNA Ladder (Solis BioDyne, Estonia), C – No template/negative control, Lanes 1–17 – Positive amplification of the *recA* gene from water and microplastic biofilm samples showing a 668bp band.

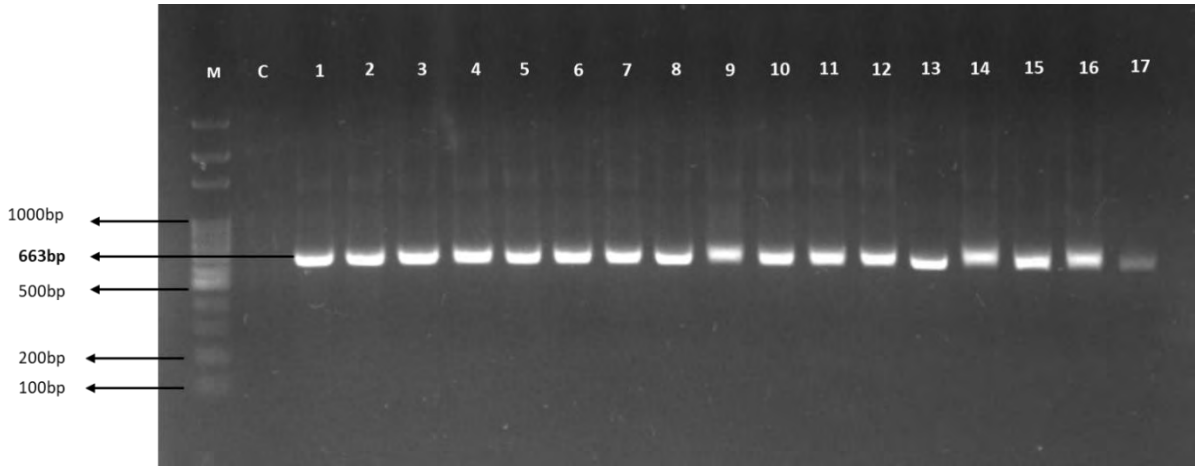

Figure 4-11. A 1% ethidium-bromide-stained agarose gels showing an instance of the detection of *Vibrio* spp. from water and microplastic biofilm samples using the *Vibrio 16S rRNA* primer. M - 100bp DNA ladder (Solis BioDyne, Estonia), C – No template/negative control, Lanes 1–17 – Positive amplification of the *Vibrio 16S rRNA* gene from water and microplastic biofilm samples, showing a 663 bp band.

4.4 DISCUSSION

In this study, novel primers targeting the *recA* gene were designed and tested both *in-silico* and experimentally for the detection of *Vibrio* spp. Their specificity, sensitivity and

amplification efficiency were compared to the widely used *Vibrio 16S rRNA* primer (V.16S-700F/1325R), using river water and microplastic biofilm samples.

Experimental comparative analysis showed that both primers exhibited broad specificity, successfully amplifying DNA from multiple *Vibrio* species and showing no amplification for non-target bacteria such as *E. coli* and *P. ascidiaceicola*. Yet, there are some clear differences between the *recA* and *16S rRNA* primers in terms of their performance characteristics and suitability for environmental detection of *Vibrio* spp.

The *16S rRNA* primer had a higher sensitivity, with a LOD down to 10^3 copies/µL, compared to the *recA* primer's LOD of 10^4 copies/µL. The *recA* primer demonstrated higher efficiency (95%) compared to the *16S rRNA* primer (85%). While optimal qPCR efficiency typically ranges between 90-110%, slightly lower efficiencies do not necessarily compromise the validity of the assay, especially when the amplification is consistent and reproducible across technical replicates. Bustin and Huggett (2017) emphasised the importance of consistency in assay performance, indicated by a strong linearity of the standard curve ($R^2 \geq 0.98$) even when the amplification efficiency falls below ideal. This is especially critical in environmental samples where inhibitors may be present. Thus, maintaining uniform conditions across samples ensures that comparisons remain meaningful even when efficiency is slightly suboptimal (Taylor *et al.*, 2010). Furthermore, when qPCR is employed for relative quantification, the absolute efficiency of primers becomes less critical, provided that all reactions are conducted under identical conditions and the efficiencies of the target and reference genes are approximately equivalent (≤5% difference) (Livak and Schmittgen, 2001; Pfaffl, 2001).

Despite its suboptimal efficiency, the *16S rRNA* primer consistently detected *Vibrio* in all water and plastic biofilm samples due to its superior sensitivity. In contrast, *recA* primer detected *Vibrio* in 19/20 (95%) water and 15/36 (42%) MP biofilm samples. Previous studies confirm the presence of *Vibrio* spp. in the Kat and Swartkops, indicating that the river conditions are suitable for the survival of *Vibrio* (Okoh *et al.*, 2015; Abioye, Osunla and Okoh, 2021). Therefore, the *recA* primer's failure to detect *Vibrio* in all water samples was unexpected. These results provide direct comparative evidence of performance in environmental matrices, demonstrating that the *recA* primer underperforms, especially in biofilms, making it less suitable for complex or low-biomass environmental samples. Nonetheless, taken together, the high efficiency and low sensitivity of the *recA* primer advocates for its use in assays with moderate to high *Vibrio* abundance levels, such as in clinical samples or food samples (following culture-based enrichment).

Sensitivity is particularly critical when working with environmental samples, due to the often low abundance of target DNA (Perez-Bou *et al.*, 2024). Highly sensitive primers are essential to detect and amplify even trace amounts of target DNA, thereby minimizing the risk of false-negatives and ensuring reliable detection within complex, low-biomass environmental matrices (Bustin and Huggett, 2017b). Literature continually supports that the *16S rRNA* gene is a more robust target for the molecular detection and/or quantification of *Vibrio* genera in environmental water samples (Caburlotto *et al*., 2012; Carvalho *et al*., 2016; Dubert *et al*., 2016; Liang *et al*., 2019; X. Wang *et al*., 2019; Laverty *et al*., 2020; Li *et al*., 2020).

The differences in gene target characteristics may have influenced the sensitivity of the primers. The *recA* gene is a single copy gene across the *Vibrio* genus and is less affected by species-level variation in gene copy number, making it a more stable proxy for estimating cell numbers (Thompson *et al.*, 2004). In contrast, the *16S rRNA* gene is a multi-copy gene, with the number of copies for *Vibrios* ranging from 7-15 within a species and between species (H. Lin *et al.*, 2018). While multi-copy targets like the *16S rRNA* gene offer enhanced sensitivity, making them highly suitable for the early detection of pathogens or surveillance in low-concentration environments, their use in absolute quantification qPCR may not directly reflect the true number of bacterial cells. This is particularly problematic when attempting to estimate microbial abundance or compare bacterial loads across different environmental samples. Absolute quantification based on multi-copy genes requires either prior knowledge of the target organism's average gene copy number or a normalisation strategy to adjust for these variables (Borchardt *et al.*, 2021; Partida *et al.*, 2025). In the absence of such calibration, estimates derived from multi-copy targets should be interpreted cautiously and may be better suited for relative quantification rather than precise enumeration.

Nevertheless, the *recA* primer remains valuable for studies targeting specific *Vibrio* strains. It can still serve as a valuable molecular microbiology tool, especially when used in conjunction with broad-range primers such as the *16S rRNA* primer to enhance detection coverage. Crisafi et al. (2011) similarly demonstrated the superior performance of the *16S rRNA* gene, compared to the *toxR* gene for the detection of *V. anguillarum* in fish tissues.

The detection of *Vibrio* in all water and plastic biofilm samples using the *16S rRNA* primer has significant public health implications. Cases of cholera and other Vibriosis have been linked to exposure through swimming, bathing or consuming contaminated water (Hirk *et al.*, 2016; Hwang *et al.*, 2020; Sacheli *et al.*, 2023). In South Africa, where cholera remains

endemic, 1395 cases and 47 related deaths were reported between February 2023 and February 2024 (World Health Organization, 2024a). Given that the rivers sampled in this study serve as critical freshwater resources for domestic, agricultural, recreation, and cultural use, the confirmed presence of *Vibrio* highlights risk of exposure to pathogens and disease transmission. There is an urgent need for continued surveillance of *Vibrio* in environmental surface waters, risk assessment and identification of risk factors for disease outbreaks.

4.5 CONCLUSION

This study assessed the performance of a newly designed *recA* primer compared to the widely used *16S rRNA* primer for detecting *Vibrio* spp. in environmental water and microplastic biofilm samples. Although both primers demonstrated good specificity, the *16S rRNA* primer demonstrated superior sensitivity and broader applicability, consistently detecting *Vibrio* even in complex samples such as plastic biofilms. These findings emphasize the importance of selecting appropriate primers in environmental molecular surveillance, with the *16S rRNA* primer being more suitable for comprehensive and rapid molecular environmental water monitoring of *Vibrio* spp. without the need for isolation of pure cultures.

CHAPTER 5: QUANTIFICATION OF *VIBRIO* SPP. AND *TETB* IN WATER AND MICROPLASTIC BIOFILMS IN URBAN RIVERS IN EASTERN CAPE, SOUTH AFRICA: ENVIRONMENTAL AND PUBLIC HEALTH IMPLICATIONS

Publications based on this chapter:

Nnadozie, H. O., Odume, N. O. and Nnadozie, C. F. Quantification of *Vibrio* spp. and *tetB* in Water and Microplastic Biofilms in Urban Rivers in Eastern Cape, South Africa: Environmental and Public Health Implications. Under review at *Scientific Reports*.

5.1 INTRODUCTION

Urban rivers often act as reservoirs for pathogenic bacteria and antibiotic resistance genes (ARGs) (Cui *et al.*, 2019; Reddy *et al.*, 2022). Within this group of pathogens, *Vibrio* spp. play a critical role in both aquaculture and human health. The genus *Vibrio* are a group of gram-negative bacteria characterised by their curved or comma-shaped cells (Sampaio *et al.*, 2022). Some *Vibrio* species cause infections in fish, molluscs and crustaceans, whereas some are human pathogens, such as *V. cholerae* which causes cholera and. *V. vulnificus*, which has a high case-fatality rate (< 50%) and is the primary cause of seafood-related fatalities worldwide (Huehn *et al.*, 2014). Globally, an estimated 1.3 to 4.0 million people are affected by cholera each year, leading to 21,000 to 143,000 deaths and most of this burden is concentrated in Sub-Saharan Africa (Ali *et al.*, 2015). As of May 31, 2024, the WHO Regional Office for Africa (AFRO) had reported 94,973 cholera cases and 1,618 deaths between February–May 2024 (World Health Organization, 2024b). In humans, *Vibrio* infections occur through contaminated drinking water or food or contact with contaminated water through open wounds (Baker-Austin *et al.*, 2018; Yin *et al.*, 2018).

An upward trend in the abundance of *Vibrio* in the aquatic environment and the incidence of cholera and *Vibrio*sis across the globe has been recorded (Baker-Austin *et al.*, 2010; Vezzulli, 2023). Deteriorating water quality caused by the discharge of waste from urban and informal settlements, industries and wastewater treatment works (WWTWs), as well as increasing surface water temperatures due to climate change, favours the abundance of *Vibrio* in freshwater (Baker-Austin *et al.*, 2017; Ford *et al.*, 2020; Archer *et al.*, 2023).

This trend of growing presence of *Vibrio* spp. coupled with their ARGs in aquatic environments, presents a significant public health risk. Tetracyclines are among the most utilised antibiotics in human, veterinary and aquaculture applications (Xu *et al.*, 2021). This has led to the extensive emergence of tetracycline resistance genes (TRGs) in aquatic environments and other environmental reservoir biofilms (Daboul et al., 2020; Deng et al., 2020; Abioye et al., 2021; Taviani et al., 2022). According to several studies, *tetB* is more prevalent in gram-negative bacteria than other TRGs (Furushita *et al.*, 2003; Roberts, 2005; Otokunefor *et al.*, 2023). In *Vibrio* species*, tetB* encodes for efflux pumps that aggressively remove tetracycline from the bacterial cell, making the bacterium resistant to the antibiotic (Roberts, 1996; Chopra and Roberts, 2001). In addition, first-generation tetracyclines frequently used as a first-line treatment for *Vibrio*-related diseases, including cholera, have been found to have *tetB* as a major resistance determinant (Ye *et al.*, 2023). Given the pressing issue of antibiotic resistance and the importance of tetracycline in treating *Vibrio*-induced diseases, focusing on *tetB* offers an important facet of our understanding of this problem.

Research has indicated that microplastics (MPs) in aquatic environments may operate as a reservoir or concentration point for *Vibrio* to the degree that plastics have higher concentrations of these bacteria than the surrounding water (Zettler et al., 2013; Zhang et al., 2020). The colonisation of plastic biofilms offers *Vibrio* benefits such as prolonged survival in the environment, access to nutrients, and dispersal to a broader geographic range (Junaid, Siddiqui, *et al.*, 2022). Similarly, ARGs may be selectively enriched in the MP biofilm through electrostatic and hydrophobic interactions (Lu, Zhang, Wu and Luo, 2019; Wu *et al.*, 2019; Wang *et al.*, 2021). Antibiotic-resistant bacteria (ARB) in MP-associated biofilms have been shown to be up to 5000 times more prevalent than those in the surrounding water column (Zhang *et al.*, 2020; Rafa *et al.*, 2024). This allows MPs to carry potential pathogens and resistance genes from one environment to another, increasing their dispersion and persistence. The interaction of *Vibrio*, ARGs like *tetB* and microplastic biofilms in aquatic environments intensifies their environmental impact.

Many of South Africa's surface and underground water systems are polluted with plastics (Mvovo, 2021; Adewuyi and Li, 2024). The Kat and Swartkops Rivers, located in Eastern Cape, South Africa, are important freshwater ecosystems that provide irrigation, recreation, traditional and religious rites, fishing and even domestic water for bathing, cooking and washing (Birkholz, 2009; Mack *et al.*, 2024). The influx of plastic waste into these rivers may create novel reservoirs that facilitate the abundance of *Vibrio* species and their ARGs. This

can complicate public health initiatives and raise the risk of waterborne illnesses. However, to date, no studies have been published on the role of plastic as a reservoir for *Vibrio* and their ARGs in these rivers (Dahms and Greenfield, 2024). This study assessed the relative abundance of *Vibrio* spp. and its tetracycline resistance gene (*tetB*) in the water column and plastic biofilms in two urban rivers in Eastern Cape, South Africa, providing essential data on the role of plastic pollution in exacerbating antibiotic resistance. Quantitative Polymerase Chain Reaction (qPCR) was used to measure the relative abundance of *Vibrio* spp. *16S rRNA* and *tetB* genes per total bacteria (via *16S rRNA* gene) in water and plastic biofilms. The abundance and types of MP debris were assessed. In addition, nutrients and environmental factors were measured to delineate key drivers influencing the abundance of *Vibrio* in the water and plastic biofilms. The study hypothesizes that MPs act as a substantial environmental reservoir, greatly enhancing the number *of Vibrio* spp. and their resistance genes, especially *tetB*, in these rivers. It is anticipated that *Vibrio* spp. will find a niche on MPs due to their surface biofilms, resulting in noticeably greater concentrations of these bacteria and related TRGs on the MPs than in the surrounding water column. The findings from this study will elucidate the drivers of resistance in urban rivers and inform mitigation of the spread of tetracycline-resistant *Vibrio* spp. in aquatic systems.

5.2 MATERIALS AND METHODS

Detailed descriptions of the study area, sites, sampling procedures, microplastic quantification and characterization methods and DNA extraction procedure are provided in Chapter 3.

5.2.1 Detection of *Vibrio 16S rRNA* and *tetB*

The presence of *Vibrio 16S rRNA* and *tetB* in the water and MP biofilm samples was detected through end-point PCR assays in 20 µL reactions using the KAPA Taq Ready Mix (Kapa Biosystems, Wilmington, MA, USA) according to the manufacturer's instructions. The *Vibrio* spp. *16S rRNA* primer developed by Tarr *et al*. (2007) and validated using 309 isolates representing 26 *Vibrio* species and 7 non-*Vibrio* species was used in this study to detect the presence of *Vibrio* (Tarr *et al.*, 2007). Its use was based on the enhanced detection capabilities demonstrated in the preceding chapter, where the primer showed superior sensitivity and broader applicability for environmental detection of *Vibrio* spp. in river water and MP biofilms, particularly compared to the newly designed *recA* primer. The *tetB* primer

developed by Han *et al*. (2015) and validated through molecular analysis of the plasmid p*TetB*-VA1 (5162 bp) (Han *et al.*, 2015) was used to detect *tetB*. The gene targets, sequences and annealing temperatures of the primers are described in Table 5-1. Additional information about the validation of the *tetB* and universal bacterial *16S rRNA* primers is available in Appendices 3 and 4.

Table 5-1. Primer names, gene targets, sequences and annealing temperatures in this study.

Primer Name	Target	Forward	Reverse	Annealing temp (˚C)	Reference
V.16S - 700F/1325R	*Vibrio 16S rRNA*	CGGTGAAA TGCGTAGA GAT	TACTAGCG ATTCCGAG TTC	60	Tarr *et al.* (2007)
tetB	*tetB*	TTGCGGGA ATTTGGCC TATCAATT	GTTGAGAC GCAATCGA ATTCGGTA T	60	Han *et al*., (2015)
515F/806R	Bacterial *16S rRNA* (V4 hypervariable region)	GTGCCAGC MGCCGCG GTAA	GGACTACH VGGGTWT CTAAT	60	Miao et al. (2019)

5.2.2 Relative quantification of *Vibrio 16S rRNA* and *tetB*

A comparative C_T method ($2^{-\Delta CT}$, where $\Delta C_T = (C_{T(\text{target gene})} - C_{T(16S\ rRNA)})$ was used to measure the relative abundance of *Vibrio 16S rRNA* and *tetB*, consistent with the qPCR methods reported in previous studies (J. Wang *et al.*, 2020; Hu *et al.*, 2021; Kim and Yoo, 2024). This method is useful for correcting sample-to-sample differences in amounts of input DNA templates. An endogenous control gene normalises the C_T values of the gene of interest by subtracting the C_T value of the endogenous control in each sample from the C_T value of the gene of interest in the same sample (Livak and Schmittgen, 2001; Zalewski, 2024). In this study, the abundance of *Vibrio* species identified through *16S rRNA* analysis,

and the gene *tetB* were normalised to the total bacterial abundance (Bacterial *16S rRNA*) in each sample to correct for the variations in the total abundance of bacteria in the MP biofilms and water samples. The resulting delta C_T values were then transformed using the $2^{-\Delta CT}$ formula and summarised using the group means. The targets and the endogenous control were amplified in separate wells with a SYBR Green approach. The qPCR was performed using the Applied Biosystems QuantStudio™ 3 System (ThermoFisher Scientific, Lenexa KS, USA). The 10 µL reactions consisted of 5.0 µL TB Green® Premix Ex Taq II (Takara Bio Inc., Kusatsu, Shiga, Japan), 0.2 µL ROX Reference Dye II (Takara Bio Inc., Kusatsu, Shiga, Japan), 0.5 µL each of the forward and reverse primer (0.5 µM), 1.8 µL ddH2O, and 2 µL DNA template. A three-step qPCR reaction was performed with the following conditions: 95ºC for 30 s, followed by 40 cycles of 95ºC for 15 s, 60ºC for 30 s and 72ºC for 60 s. Fluorescent signals were captured in the elongation step. The specificity of the reaction was verified by an evaluation of the melt curves of the amplification products, which were obtained after the completion of the PCR cycles using an additional thermal step (95ºC for 15 s, 60ºC for 60 s, and 95ºC for 1 s). Each sample and non-template reaction control (NTC) was run in triplicate. A threshold cycle (C_T) of 40 was used as the detection limit. Primer amplification efficiency was assessed by evaluating the slope and R^2 values of the standard curve prepared from a qPCR run using standards of each target gene, with concentrations ranging from 10^8 to 10^3 gene copies per reaction (Table 5-2).

Table 5-2. Standard curve, R^2 and amplification efficiency of the target genes and endogenous control

Target gene	Standard curve	R^2	Efficiency (%)
Bacterial *16S rRNA*	-3.5864x + 32.114	0.997	90
Vibrio 16S rRNA	-3.748x + 33.151	0.999	85
tetB	-3.6034x + 32.522	0.999	89

5.2.3 Statistical analysis

Normality and homoscedasticity of the data for *Vibrio 16S rRNA* and *tetB* abundances and nutrient concentrations were verified using the Shapiro–Wilk and Levene's tests, respectively, to inform the choice of statistical tests and enhance the reliability and robustness of the statistical analyses. The data were log10 transformed before analyses due to their non-normal distribution. *Vibrio 16S rRNA* and *tetB* abundances were analysed

using descriptive statistics and plotted using the statistical software R version 4.4.1, including the package ggplot2. The independent Student t-test was used to analyse the differences in *Vibrio 16S rRNA* and *tetB* abundances between water and MP biofilm and between the two rivers. Correlations between MPs, ARGs, *Vibrio 16S rRNA* genes and physicochemical parameters were assessed with Spearman's rank correlation analysis. Correlation coefficients ($r < 0.3$ or <-0.3) were considered to either be very weak or a non-correlation, $r > 0.3 \leq 0.5$ were regarded as moderate and $r > 0.5$ to be strong correlations (Taylor, 1990). All statistical tests were considered significant at $P < 0.05$. All the analyses mentioned were performed using R version 4.4.1.

5.3 RESULTS

5.3.1 Physicochemical parameters

The temperature, pH and dissolved oxygen (DO) of both rivers were similar. The average temperature was 18.87 ± 5.17°C in the Swartkops River and 16.70 ± 6.55 in the Kat River. The mean DO concentration in the Kat was 3.77 ± 2.27, and in Swartkops, 2.08 ± 1.10. The average pH in both rivers was alkaline, with the mean pH of the Kat River (9.79 ± 2.63) slightly higher than that of the Swartkops (8.60 ± 1.44). The highest pH value recorded during the study period was 14.00, which was recorded in sites K3 and K4, which are influenced by run-off from nearby commercial-scale citrus farms. The average turbidity in the Kat River (42.18 ± 29.56 NTU) was higher than in the Swartkops (10.69 ± 7.73 NTU). In both rivers, the sites downstream of WWTW discharge points (S2 and K2) exhibited the highest turbidity. Salinity was low overall, however, the average salinity in the Swartkops River (1.26 ± 0.84 ppt) was higher than in the Kat River (0.07 ± 0.08 ppt). Nutrient levels were higher in the Swartkops than in the Kat River. In the Swartkops, the average concentration of Nitrates, Nitrites, Ammonia and Phosphates were 14.30 ± 10.29 mg/L, 0.35± 0.47 mg/L, 2.05± 2.16 mg/L and 4.89± 3.05 mg/L, respectively. On the other hand, the average concentration of Nitrates, Nitrites, Ammonia and Phosphates in the Kat River were 3.43± 2.52 mg/L, 0.10± 0.04 mg/L, 0.35± 0.15 mg/L and 0.88± 0.48 mg/L, respectively (Table 5-3).

Table 5-3. Physicochemical properties of the Swartkops and Kat rivers during the study period

Environmental Variables	Swartkops (N=12)		Kat (N=8)	
	Mean ± SD	**Range (Min-Max)**	**Mean ± SD**	**Range**
Temperature (°C)	18.87 ± 5.17	13.27–25.18	16.70 ± 6.55	9.36–24.24
pH	8.60 ± 1.44	7.18–11.20	9.79 ± 2.63	7.65–14.00
Salinity (ppt)	1.26 ± 0.84	0.00–2.45	0.07 ± 0.08	0.00–0.22
Dissolved oxygen (mg/L)	2.08 ± 1.10	0.48–4.71	3.77 ± 2.27	0.00–6.42
Turbidity (NTU)	10.69 ± 7.73	3.59–24.70	42.18 ± 29.56	9.73–89.00
Nitrate (mg/L)	8.35 ± 9.54	0.31–28.14	2.26 ± 2.17	0.50–6.52
Nitrite (mg/L)	0.29 ± 0.38	0.01–1.15	0.09 ± 0.04	0.03–0.14
Phosphate (mg/L)	2.99 ± 2.98	0.01–9.77	0.49 ± 2.55	0.07–1.43
Ammonia (mg/L)	1.98 ± 2.18	0.00–6.06	1.25 ± 0.52	0.05–7.97

5.3.2 MP abundance and classification

Four distinct shapes of MPs were identified: fragments, fibres, films and pellets (Figure 5- 1). All sites showed MP pollution. The MP count ranged from 69 (S1) to 403 particles (S2) in the Swartkops and 74 (K1) to 220 particles (K2) in the Kat River. Notably, in both rivers, the sites downstream of WWTW discharge points had the highest MP count (S2 and K2). The average MP abundance was estimated to be 1.55 particles/L in the Kat River and 1.52 particles/L in the Swartkops River. In the Swartkops, the abundance of different types of MP was fragment > fibre > film > pellet. In the Kat, the MP abundance was fragment > film > fibre > pellet. MP abundance and classification data are given in Table 5-4.

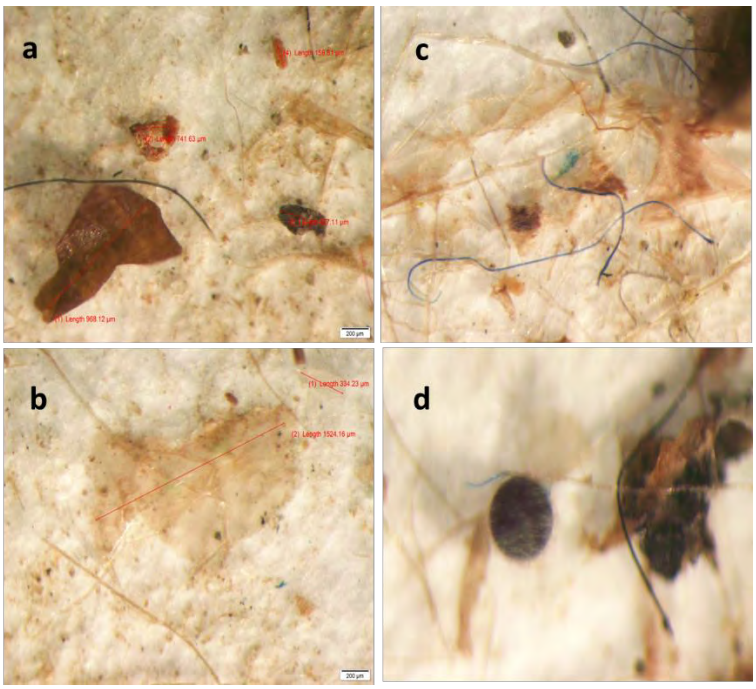

Figure 5-1. Representative MP particles from environmental samples, photographed with a Stereo zoom microscope and classified based on shape/appearance: A – Pellet, B – Fibres, C – Film, D – Fragment.

Table 5-4. Count and abundance of different MP types from 10 sites in the Kat and Swartkops rivers during the sampling period

Sample ID	Fragment	Film	Fibre	Pellet	Count total	Abundance (particles/L)
Kat river						
K1	24	22	27	1	74	0.74
K2	105	57	56	2	220	2.20
K3	47	51	11	0	109	1.09
K4	154	32	29	0	215	2.15
Swartkops river						
S1	21	23	23	2	69	0.69
S2	119	85	196	3	403	4.03
S3	68	20	34	1	123	1.23
S4	34	3	52	2	91	0.91
S5	79	8	32	0	119	1.19
S6	44	17	43	0	104	1.04

5.3.3 Detection of *Vibrio* and *tetB* genes

A total of 12 water and 22 MP biofilm samples were analysed from 6 sites in the Swartkops River, while 8 water and 14 MP biofilm samples were analysed from 4 sites in the Kat River. For both the Swartkops and Kat Rivers, positive amplification of *Vibrio 16S rRNA* was detected in 100% of water and MP biofilm samples. *tetB* was detected in 6 (50%) water and 17 (77%) MP biofilm samples from the Swartkops River, and 6 (75%) water and 7 (50%) plastic biofilm samples from the Kat River. Sequence comparison of the amplicons using the BLASTN sequence similarity search in the NCBI database confirmed positive detection of the *Vibrio 16S rRNA* and *tetB* genes. The *V16S rRNA* amplicons showed between 80–87% sequence identity to various *Vibrio* spp. 16S ribosomal RNA with a 99–100% query cover. The *tetB* amplicons showed 95–96% sequence identity to various *Vibrio* spp. plasmid and *tetB* genes with a 91% query cover.

5.3.4 Relative abundance of *Vibrio 16S rRNA* and *tetB* genes

Forty-three samples were analysed for *Vibrio 16S rRNA* relative abundance, including 11 water and 13 MP biofilm samples from 6 sites in the Swartkops and 8 water and 11 MP biofilm samples from 4 sites in the Kat River. Additionally, *tetB* relative abundance was assessed in 45 samples, 11 water and 16 MP biofilm samples from 6 sites in the Swartkops and 8 water and 10 MP biofilm samples from 4 sites in the Kat River.

The observed differences in the relative *Vibrio 16S rRNA* abundance and *tetB* between water and MP samples were not statistically significant (Student's t-test, $P > 0.05$). However, interpretation should focus on the observed patterns due to the limited sample size per gene and site. According to Figures 5-2 and 5-3, higher maximum values of the relative *Vibrio 16S rRNA* abundance were generally observed in water, a pattern consistent for both rivers. Microplastic biofilms, however, exhibited higher maximum values of the relative *tetB* gene abundance across both rivers.

Observed spatial trends revealed notable site-specific variations in relative gene abundances. In the Swartkops River, the water sample with the highest relative abundance of *Vibrio 16S rRNA* was collected from S2, downstream of a wastewater treatment works (WWTW) discharge point (Figure 5-4a), whereas MP biofilms at S4, situated in a highly industrial urban area, had the highest abundance (Figure 5- 4b). Similarly, S2 water samples showed the highest relative abundance of *tetB* (Figure 5-5a), while MP biofilms at S4 exhibited the highest relative *tetB* abundance (Figure 5-5b). In the Kat River, the highest

relative abundances of both *Vibrio 16S rRNA* were observed in water samples from K3 and MP biofilm samples from K4. Both sites were surrounded by commercial citrus farms. Meanwhile, *tetB* relative abundance was highest in the water and MP biofilm samples from K2, downstream of a WWTW discharge point (Figures 5-4a, 5-4b, 5-5a, 5-5b).

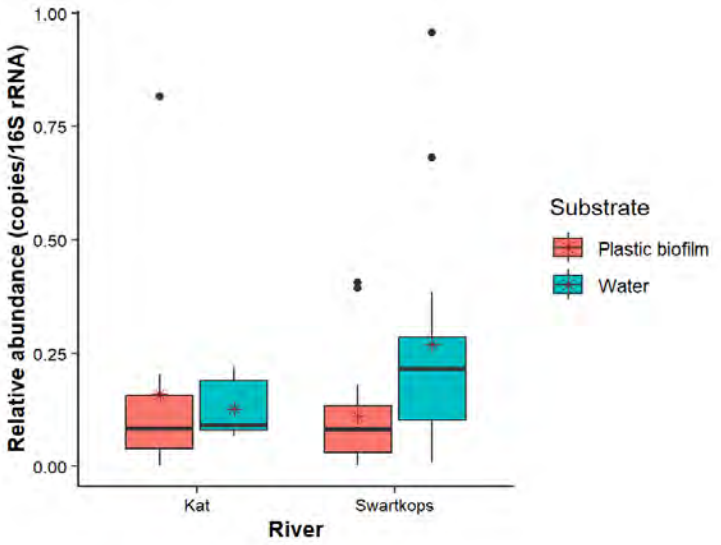

Figure 5-2. Relative abundance (Number of *Vibrio 16S rRNA* gene copies per total bacterial *16S rRNA* gene copies) in water and MP biofilm samples collected from the Kat and Swartkops rivers between March and July 2023. The boxes and whiskers indicate the interquartile range and variability of the relative abundance data within each substrate group; the black lines inside the boxes indicate the median values, and the dark-red asterisks indicate the mean relative abundance in each group.

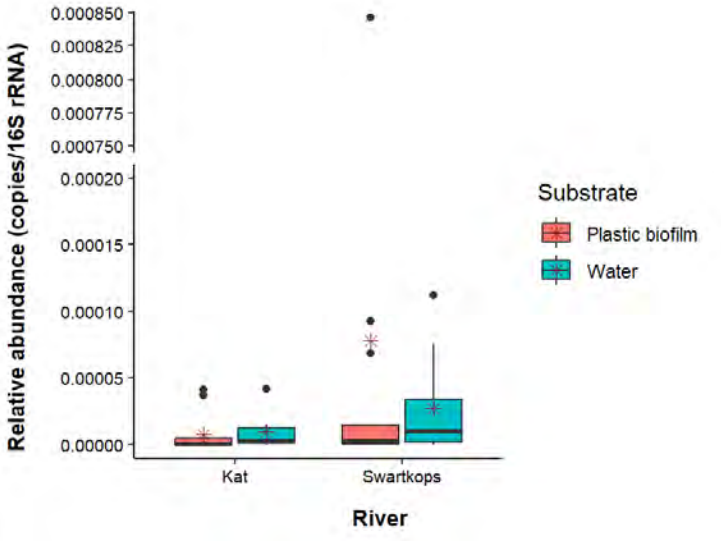

Figure 5-3. Relative abundance (Number of *tetB* gene copies per total bacterial *16S rRNA* gene copies) in water and MP biofilm samples collected from the Kat and Swartkops rivers

between March and July 2023. The boxes and whiskers indicate the interquartile range and variability of the relative abundance data within each substrate group; the black lines inside the boxes indicate the median values, and the dark-red asterisks indicate the mean relative abundance in each group.

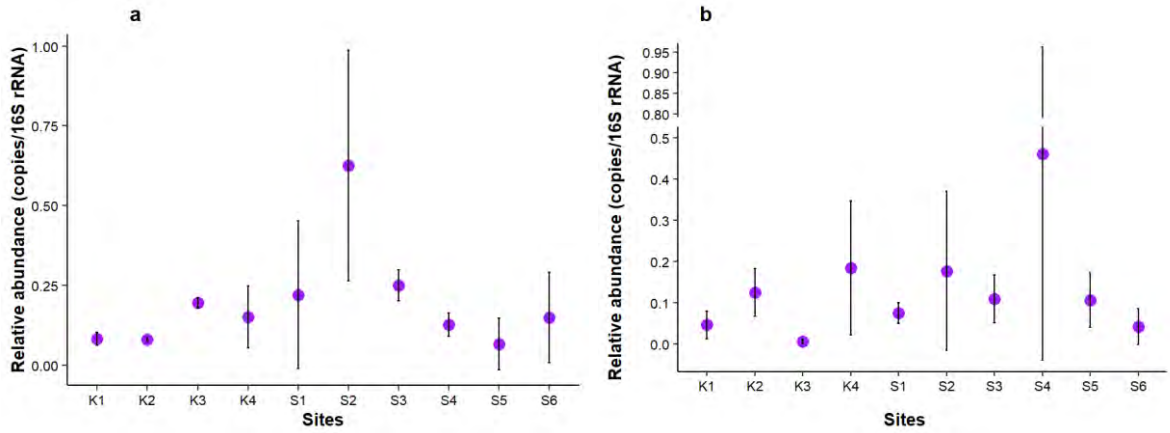

Figure 5-4. Relative abundance of the *Vibrio 16S rRNA* gene (Number of *Vibrio 16S rRNA* gene copies per total *bacterial 16S rRNA* gene copies) **a)** in water and **b)** in MP biofilm samples collected from sites along the Kat (Sites K1–K4) and Swartkops (Sites S1–S6) rivers, respectively. The points indicate the mean values, while the horizontal lines indicate the standard deviation calculated from the replicate samples collected from each site between March and July 2024.

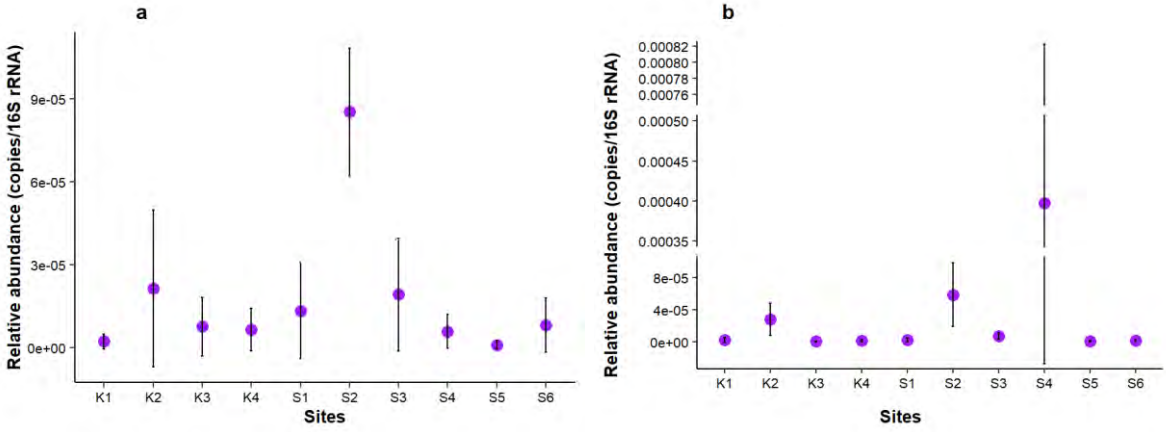

Figure 5-5. Relative abundance of the *tetB* gene (Number of *tetB* gene copies per total *bacterial 16S rRNA* gene copies) **a)** in water and **b)** in MP biofilm samples collected from sites along the Kat (Sites K1–K4) and Swartkops (Sites S1–S6) rivers, respectively. The points indicate the mean values, while the horizontal lines indicate the standard deviation calculated from the replicate samples collected from each site between March and July 2024.

5.3.5 Relationships Between the Abundance of *tetB* and *Vibrio 16S rRNA* Genes and Their Correlations with Water Quality Parameters

In both rivers, the relative abundance of *Vibrio 16S rRNA* showed a strong positive correlation to the abundance of *tetB*, although the association was significant only for the Swartkops River (r=0.943, p=0.017) and not for the Kat River (r= 0.800, p=0.333). On the other hand, there were no significant correlations between MP distribution and relative abundance of *Vibrio 16S rRNA* (Swartkops r=0.314, p=0.564; Kat r=0.800, p=0.333) or *tetB* (Swartkops r= 0.086, p=0.919; Kat r=1.000, p=0.083).

Spearman's correlation analysis revealed different strengths and directions of correlations that highlight the influence of environmental factors on the relative abundance of *Vibrio* and *tetB* in the two rivers. In the Swartkops River, dissolved oxygen and turbidity showed the strongest positive correlations (r=0.385, p=0.047 and r=0.565, p=0.002) with *tetB*. *tetB* exhibited moderate but non-significant correlations with pH and nitrite, while its correlation with salinity, nitrate, and phosphate was negligible. For *Vibrio 16S rRNA*, none of the correlations were significant, except for nitrites (r=0.372, p=0.073), which exhibited a moderate positive correlation (Table 5-5). In the Kat River, *tetB* relative abundance exhibited positive but non-significant correlation with nitrates (r=0.404, p=0.097) and phosphates (r=0. 376, p=0.124), while correlations with nitrites (r=-0.336, p=0.173) and pH (r=-0.198, p=0.430) were negative and weak (Table 5-5). Finally, turbidity showed a moderate positive relationship with *tetB* (r=0.347, p=0.159). *Vibrio 16S rRNA* relative abundance negatively correlated with ammonia (r=-0.444, p=0.057); however, no other parameter showed significant associations.

Table 5-5. Spearman correlation of *Vibrio 16S rRNA* and *tetB* relative abundance and environmental parameters in the Swartkops and Kat Rivers during the study period[a]

Environmental variable	**Swartkops**		**Kat**	
	Vibrio 16S	***tetB***	***Vibrio 16S***	***tetB***
Temperature (°C)	0.215	0.021	-0.142	0.307
	(0.312)	(0.919)	(0.563)	(0.215)
pH	-0.097	0.370	0.176	-0.198
	(0.651)	(0.057)	(0.470)	(0.430)
Salinity (ppt)	0.202	-0.196	-0.193	-0.042
	(0.344)	(0.328)	(0.430)	(0.869)

Dissolved oxygen (mg/L)	-0.078	0.385	0.032	0.211
	(0.717)	**(0.047)**	(0.879)	(0.40)
Turbidity (NTU)	0.106	0.565	-0.088	0.347
	(0.621)	**(0.002)**	(0.719)	(0.159)
Nitrate (mg/L)	0.076	-0.252	-0.358	0.404
	(0.723)	(0.204)	(0.132)	(0.097)
Nitrite (mg/L)	0.372	0.295	-0.281	-0.336
	(0.073)	(0.135)	(0.243)	(0.173)
Phosphate (mg/L)	0.278	-0.229	-0.025	0.376
	(0.189)	(0.250)	(0.920)	(0.124)
Ammonia (mg/L)	0.204	0.303	-0.444	0.172
	(0.340)	(0.125)	(0.057)	(0.496)

[a]| The table shows the Spearman correlation coefficients (r) between 1 and -1 and corresponding p values (in parentheses). A negative r value indicates a negative correlation, while a positive value indicates a positive correlation. $p < 0.05$ indicates a significant correlation. Significant correlations are indicated in bold.

5.4 DISCUSSION

This study assessed the associations between environmental factors, MP debris, *Vibrio 16S rRNA* and the *tetB* gene across two river systems in Eastern Cape, South Africa. Rather than providing simple cause-and-effect associations, the findings highlight the multifaceted influences which control microbial abundance and antibiotic resistance distribution in aquatic ecosystems (Amaral-Zettler et al., 2015; Oberbeckmann et al., 2016; Laverty et al., 2020; Coons et al., 2021; Vincent et al., 2022).

Similar temperature, pH and dissolved oxygen profiles were detected in the physicochemical characterisation of both rivers, indicating similar site conditions across the rivers. The environmental conditions of the rivers in this study (Table 5-3) fall within the range of those reported in previous studies (temperature: <0–27°C, salinity: 0.06–34 ppt, pH: 6–10, turbidity: 2.16–130 NTU, nitrates: 0–29 mg/L, nitrites: 0.01–2.06 mg/L, phosphates: 0.03–3.69 mg/L, ammonia: 0.06–96.87 mg/L), all of which are conducive to the survival of *Vibrio* spp. (Igbinosa et al., 2011; Johnson et al., 2012; Böer et al., 2013; Kopprio et al., 2017; Liang et al., 2019; X. Wang et al., 2020; Abioye et al., 2021).

A thorough correlation analysis revealed nuanced associations: *Vibrio 16S rRNA* abundance and *tetB* levels in the Swartkops River exhibited a significant correlation ($r = 0.943$, $p = 0.017$), but this tendency did not hold true for the Kat River ($r = 0.800$, $p = 0.333$). Lower sample sizes per site and site-specific variations in environmental variables could be the cause of this discrepancy (Osenberg *et al.*, 1994). Additionally, the *tetB* gene abundance observed in this study may have been linked to the specific *Vibrio* species present in the study sites. However, the lack of species-specific enumeration of *Vibrio* spp. in this study limits the interpretation of *tetB* abundances. Further studies involving species-specific PCR or metagenomic sequencing may elucidate the relationship between the *Vibrio* community composition and *tetB* abundance. The significant correlation observed in the Swartkops River aligns with other studies reporting a consistent association of *tetB*, and microbes such as *Vibrio* spp. have been observed in various environments (Furushita *et al.*, 2003; Otokunefor *et al.*, 2023).

The abundance and types of MP debris were also assessed as potential drivers. Swartkops had an average MP concentration of 1.52 particles/L, while the Kat had an average of 1.52 particles/L. This is the first time the quantity of MPs in these rivers has been documented. The mean abundance of MPs in these rivers is higher than reported in other South African rivers (e.g., Vaal, Mooi, Wasgoedspruit Rivers) (Bouwman *et al.*, 2018), and in various international rivers such as the Soar, Leen and Trent Rivers, United Kingdom (0.019 to 0.4 particles/L) (Stanton *et al.*, 2020), Lake Victoria, East Africa (0.00073 particles/L) (Egessa *et al.*, 2020) and the Ofanto river in Italy (0.0009 to 0.013 particles/L) (Campanale, Stock, *et al.*, 2020). However, compared to other freshwater systems like the Yangtze, Pearl and Rhine Rivers (Zhao *et al.*, 2014; Mani *et al.*, 2015; L. Lin *et al.*, 2018), the observed MP concentrations fall within the lower-to-medium range. The observed MP loads in these rivers point to local anthropogenic pressures contributing to contamination in Eastern Cape rivers. Concurring with other studies (Eriksen *et al.*, 2013; McCormick *et al.*, 2014; Campanale, Stock, *et al.*, 2020), MPs were more concentrated at sites downstream of wastewater treatment works (WWTW) discharges (Table 5-4).

Microplastics may indirectly act as reservoirs by forming microniches that promote gene persistence (McCormick *et al.*, 2014; Wu *et al.*, 2019; Wang *et al.*, 2021). The selective enrichment of microorganisms and ARGs may be explained by the stability of MP-associated biofilms in contrast to transient water environments (Laverty *et al.*, 2020; Bae and Yoo, 2022). However, in this study, they exhibited no significant correlation with the abundance of either *Vibrio 16S rRNA* or *tetB*. This finding raises questions about the

presumed role of MPs as consistent vectors of microbial resistance genes in riverine systems (J. Wang *et al.*, 2020; S. Wang *et al.*, 2020). The lack of a direct correlation between MP concentrations and gene abundance implies that this link is mediated by land-use patterns and external pollution sources (Oberbeckmann et al., 2018; Xue et al., 2020; Vincent et al., 2022). Site-specific drivers such as turbidity, dissolved oxygen and proximity to effluent discharge appear to play more dominant roles. For example, the moderate correlation between turbidity and *tetB* in Swartkops ($r = 0.565$, $p = 0.002$), although it was not mirrored in the Kat River, reinforces the role of localized conditions in shaping microbial distributions.

A key factor in the patterns observed is the significant impact of human activity, particularly wastewater inputs. *Vibrio 16S rRNA* and *tetB* levels were consistently higher at sites close to WWTW discharge stations. This emphasises how important it is to better manage wastewater to prevent the spread of harmful microbes and resistance genes in freshwater environments (Igbinosa et al., 2011; Aubertheau et al., 2017; Okeyo et al., 2018).

Previous studies have shown that changes in flow rate may have an impact on sorption and transport processes (Lehtola et al., 2006; Zhang et al., 2023), while co-selectors such as heavy metals, antibiotics or other contaminants may be the source of indirect selection pressures (Xue *et al.*, 2020; Wang *et al.*, 2021; Q. Li *et al.*, 2022; Zhang *et al.*, 2022), influencing the abundance of microorganisms and ARGs on MP biofilms. Further understanding of the reported microbial and resistance patterns could be achieved by examining additional factors such as co-selectors, the presence of antibiotics and flow rates that may have influenced these results, as these were not directly measured in this study.

5.5 CONCLUSION

Microplastics are present in both rivers studied, with a higher abundance in the Swartkops River. The water and MP biofilms in the Swartkops and Kat Rivers can act as reservoirs of ARGs (*tetB*) and *Vibrio* spp. and may promote the persistence of these genes. However, in this study, no direct association was found between the MPs and the abundance of *Vibrio* spp. or their resistance genes. This study revealed that sampling sites downstream of wastewater treatment works (WWTW) effluent had higher levels of microplastics, *Vibrio* spp., and their ARGs. This research emphasises the importance of effectively monitoring the standard of WWTW effluents to protect rivers from harmful pollutants. Additionally, this

study showed that environmental conditions and pollution levels are important factors that affect the occurrence and spread of these bacteria.

CHAPTER 6: GENERAL DISCUSSION AND CONCLUSION

6.1 INTRODUCTION

This chapter provides an overview of the key findings from this study, discussing their broader implications, contributions to the field and limitations. It also offers recommendations for future research and concludes with a summary of the overall findings. The objectives of this study were to 1) investigate existing literature on ARB and ARG enrichment on microplastics (MPs) in aquatic environments, including *Vibrio* spp. and *tetB*, 2) assess the contamination levels of MP debris in selected urban rivers of Eastern Cape, South Africa and 3) detect, quantify and compare the abundance of *Vibrio* spp. and *tetB* in MP-associated biofilms and surrounding water. Additionally, this chapter discusses the ecological and public health implications of the current study, its limitations, and recommendations for future research.

6.2 SUMMARY OF KEY FINDINGS

The study aimed to assess the role of microplastic-associated biofilms in the enrichment of *Vibrio* spp. and *tetB* in urban rivers of Eastern Cape, South Africa, and their potential role in the persistence of antibiotic resistance and waterborne infections. To achieve this, the study conducted a comprehensive literature review to identify knowledge gaps and assess the role of MPs as reservoirs of ARBs and ARGs in aquatic environments, as well as inform the design and methodology of this study. It also designed and tested a novel *recA* primer and compared its performance to the widely used *16S rRNA* primer (V.16S-700F/1325R) in terms of specificity, sensitivity and efficiency. The most effective primers were then used to conduct quantitative analyses to measure the relative abundance of *Vibrio 16S rRNA* and *tetB* genes in river water and MP biofilm samples.

The literature review revealed that there is no clear consensus on whether MP biofilms consistently enrich bacterial pathogens or ARGs in aquatic environments. While numerous studies focus on MP abundance in marine environments, research on their occurrence in freshwater systems is limited. Results from this study showed that MP abundance in the Swartkops River ranged from 0.69 to 4.63 particles/L, with a mean of 1.52 particles/L, while in the Kat River it ranged from 0.74 to 2.20 particles/L, with a mean of 1.55 particles/L. The

relative abundance of *Vibrio 16S rRNA* ranged from 9.46 x 10^{-3} to 1.86 copies/*16S rRNA* in water and 4.66 x 10^{-3} to 0.82 copies/*16S rRNA* in MP biofilms. *tetB* relative abundance ranged from 1.60 x 10^{-8} to 1.55 x 10^{-4} copies/*16S rRNA* in water and 9.51 x 10^{-8} to 8.46 x 10^{-4} copies/*16S rRNA* in MP biofilms. The study further found that anthropogenic sources, particularly wastewater treatment works (WWTWs), were the primary drivers of bacterial and ARG abundance, surpassing the influence of MP presence. Contrary to previous assumptions, MPs did not significantly enrich bacterial pathogens or ARGs compared to the surrounding water. These findings highlight the complex interactions between anthropogenic activities, microbial communities, ARGs and environmental pollution, contributing to a deeper understanding of their distribution in aquatic ecosystems.

6.3 CONTRIBUTIONS TO THE FIELD

This study makes several important contributions to environmental monitoring and microbial ecology. The use of quantitative analysis (qPCR) offered essential insights into the distribution of *Vibrio 16S rRNA* and *tetB* genes, highlighting that anthropogenic sources exert a greater influence on bacterial and ARG abundance than microplastic presence alone. By directly comparing abundance of *Vibrio 16S rRNA* and *tetB* genes in water and MP biofilms, this study clarified the role of MP-associated biofilms in bacterial and ARG distribution, challenging the widely held assumption that MPs universally enrich pathogens and resistance genes in aquatic environments.

Another important contribution is the development of a novel *recA* primer for *Vibrio* spp. This primer expands the molecular tools available for detecting this important bacterial genus, which is associated with both environmental and public health risks. While the *recA* primer exhibited specificity in detecting *Vibrio* spp., experimental results demonstrated that the *16S rRNA* primer had broader applicability and higher sensitivity. The comparison between these two primers provides valuable insights for researchers selecting molecular tools for environmental monitoring.

6.4 STUDY LIMITATIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

Although this study provides valuable insights, certain limitations must be acknowledged. One limitation is that absolute bacterial loads were not quantified through qPCR, which may limit the precision of microbial density estimates. The limited temporal scope of the study is

another constraint, as samples were collected only twice during the study period. Because of this, temporal variations in *Vibrio* spp. and *tetB* abundance, and the environmental variables such as temperature, salinity and nutrient levels, which are known to influence bacterial colonisation and biofilm formation on MPs could not be extensively assessed. The lack of significant enrichment of *Vibrio* and *tetB*, as well as the absence of correlations between gene abundances and environmental conditions, may be attributed to this limitation. The presence of antibiotics in the samples was not screened for in this study. Previous studies have found that antibiotics contamination of aquatic environments significantly enriches ARBs and ARGs (Xue *et al.*, 2020; Wang *et al.*, 2021; Q. Li *et al.*, 2022; Zhang *et al.*, 2022). These constraints highlight areas where future research could enhance and build upon the findings of this study.

Building on the findings and limitations of this study, the following recommendations are proposed. Future research should incorporate metagenomic and functional assays to provide a more comprehensive understanding of microbial communities and ARG distribution within aquatic systems. Expanding the analysis to include additional ARGs would also be beneficial, as it would allow for a more complete evaluation of antibiotic resistance patterns in these environments. Furthermore, culturing ARG-positive samples on selective media could confirm which species/plasmids carry the resistance genes.

Investigating the role of anthropogenic sources, such as wastewater discharge, agricultural runoff and industrial pollution in shaping bacterial persistence and ARG dissemination could provide critical insights into the environmental drivers of their persistence. This research could guide future studies aimed at developing strategies to mitigate the spread of antibiotic resistance in the environment.

Future studies might benefit from a long-term sampling or in-situ incubation approach to assess the enrichment of *Vibrio* and ARGs on microplastics over time. This would allow for a more robust evaluation of the enrichment of *Vibrio* and ARGs in the microplastisphere and enable the assessment of the long-term temporal variations in environmental conditions, as well as their impacts on *Vibrio* spp. and ARG abundance in these rivers.

6.5 CONCLUSION

This study provides valuable insights into MP contamination and its role in the persistence of *Vibrio* spp. and *tetB* persistence in the Kat and Swartkops rivers, Eastern Cape, South Africa. The development of a new *recA* primer render enhanced detection capabilities, while

qPCR analysis reveals the complex distribution of microbes in aquatic environments. *Vibrio* spp. and *tetB* were detected in both MP biofilms and the surrounding river water. Unlike some previous studies, these findings do not support the assumption that MPs commonly enrich bacterial pathogens and ARGs. Instead, anthropogenic sources such as wastewater discharge, agricultural runoff and industrial activities emerged as key drivers of microbial contamination and antibiotic resistance in these rivers. These findings emphasise the need for comprehensive pollution management strategies that extend beyond MPs to address broader environmental contributors to antibiotic resistance. Future research should further investigate the ecological and public health implications of anthropogenically-driven microbial changes and develop improved monitoring and mitigation approaches for antibiotic resistance in aquatic systems. By addressing these challenges, we can contribute to more effective policies for safeguarding environmental and public health.

This study also confirms the superior sensitivity and broader applicability of the *16S rRNA* primer in detecting *Vibrio* spp., while acknowledging the *recA* primer's potential for targeted applications. The knowledge gained from this study contributes to a growing body of data on microbial ecology, antibiotic resistance and environmental monitoring, paving the way for future risk assessment strategies.

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APPENDICES

Appendix 1: Ethical Clearance

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Rhodes University Human Research Ethics Committee
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7 March 2023

Dr Chika Nnadozie

Email: C.Nnadozie@ru.ac.za

Review Reference: 2022-4864-7324

Dear Dr Chika Nnadozie

Re: Human ethics renewal application: Investigating the multiple risk dimensions associated with Campylobacteriosis - a key poverty-related disease of South African urban source water environments

Researcher: Dr Chika Nnadozie

Supervisors: ,

This letter confirms that the above Annual Report has been reviewed and **APPROVED** by the Rhodes University Human Research Ethics Committee (RU-HREC). Your Approval number is: 2022-4864-7324

Approval has been granted for 1 year. An annual progress report will be required in order to renew approval for an additional period.

Please ensure that the Human Research Ethics Committee is notified should any substantive change(s) be made, for whatever reason, during the research process. This includes changes in investigators. Please also ensure that a brief report is submitted to the ethics committee on the completion of the research. The purpose of this report is to indicate whether the research was conducted successfully, if any aspects could not be completed, or if any problems arose that the Human Research Ethics Committee should be aware of. If a thesis or dissertation arising from this research is submitted to the library's electronic theses and dissertations (ETD) repository, please notify the committee of the date of submission and/or any reference or cataloguing number allocated.

Sincerely,

Dr Janet Hayward

Chair: Rhodes University Human Research Ethics Committee, RU-HREC

cc: Ethics Coordinator

Page 1 of 1

Figure A1-1. Ethical clearance letter for this study. Ethical clearance was received from the Rhodes University Human Research Ethics Committee.

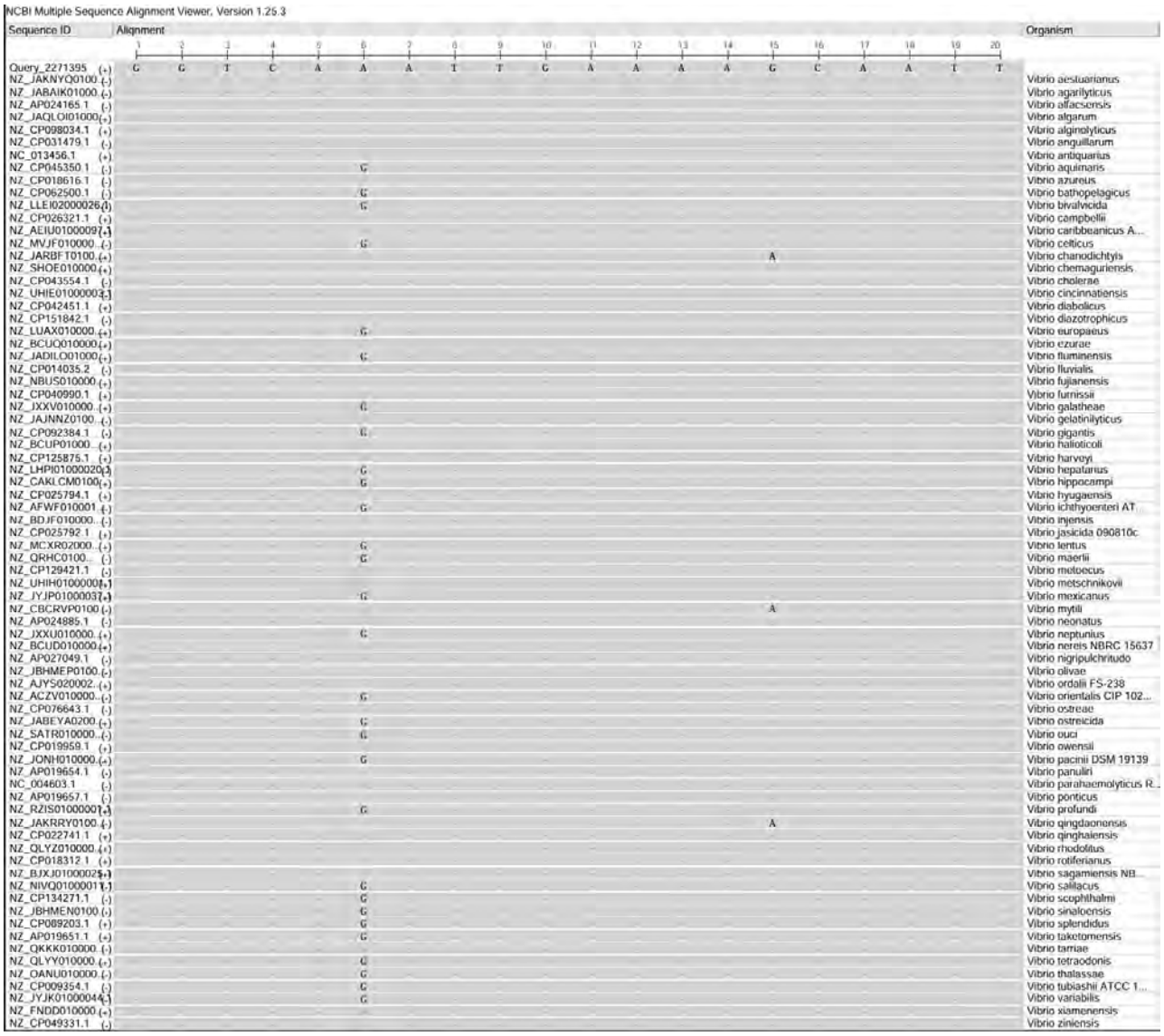

Figure A2-1. Alignments of the forward primer to the corresponding regions in various *Vibrio* spp. The specificities of primers for *Vibrio* are determined by mismatches at the different nucleotide sites, especially at 3' termini.

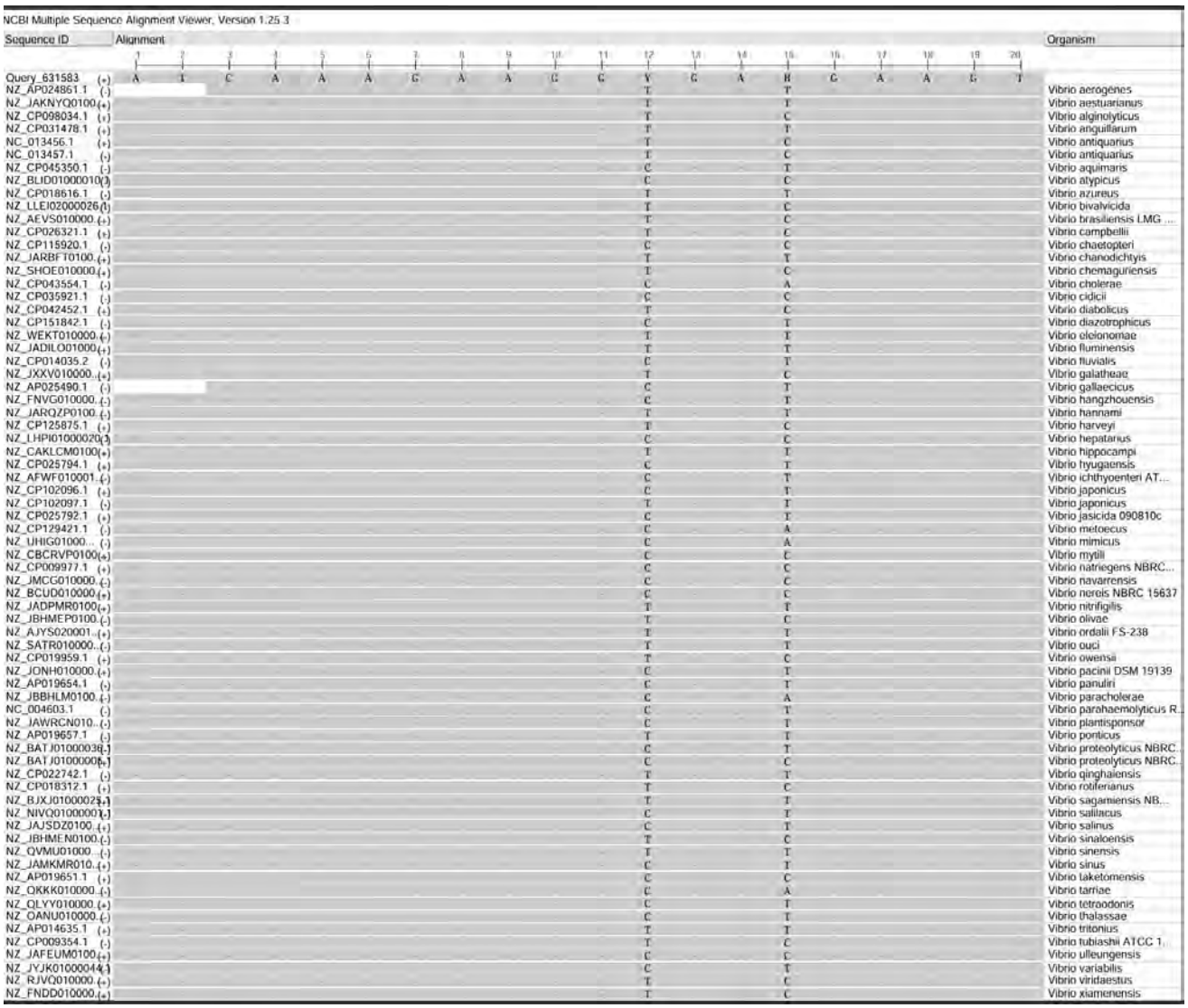

Figure A2-2. Alignments of the reverse primer to the corresponding regions in various *Vibrio* spp. The specificities of primers for *Vibrio* are determined by mismatches at the different nucleotide sites, especially at 3' termini.

Appendix 3: *tetB* primer validation and detection of *tetB* from water and microplastic biofilm samples

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- Amplification efficiency testing

The efficiency of the *tetB* primer was determined in one qPCR run with amplicons generated from environmental water samples in six serial dilutions, ranging from 10^9 to 10^4 gene copies/µL, in triplicate. qPCR was performed using the Applied Biosystems QuantStudio™ 3 System (ThermoFisher Scientific, Lenexa KS, USA). The 10 µL reaction consisted of 5.0 µL TB Green® Premix Ex Taq II and 0.2 µL ROX Reference Dye II (Takara Bio Inc., Kusatsu, Shiga, Japan), 0.5 µL each of the forward and reverse primer (0.5 µM), 1.8 µL ddH2O and 2 µL DNA template. A three-step qPCR reaction was performed with the following conditions: 95ºC for 30 s, followed by 40 cycles at 95ºC for 15 s, 60ºC for 30 s and 72ºC for 60 s. Fluorescent signals were captured in the elongation step. The specificity of the reaction was verified by an evaluation of the melt curves of the PCR products, which were obtained after the completion of the qPCR cycles using an additional thermal step (95ºC for 15 s, 60ºC for 60 s, and 95ºC for 1 s). Each concentration and non-template reaction control (NTC) was run in triplicate. The efficiency of the primer was calculated with the equation E = (10^-1/slope - 1) *100%. Fluorescent signals remained undetermined via qPCR in the reactions with a concentration of 10^9 gene copies, potentially due to the high concentration of the template DNA exceeding the equipment sensitivity, indicating the upper limit of quantification (LOQ) for the Applied Biosystems QuantStudio™ 3 System (ThermoFisher Scientific, Lenexa KS, USA). Positive signals were observed for concentrations between 10^8 to 10^4, with varying C_q values. A plot of the C_q values versus the logarithm of the gene copy number resulted in a regression coefficient (R^2) of 0.999, with a slope of -3.60, corresponding to an efficiency of 89% (Figure A3-1). Melt curve analysis showed a single peak around 78.82 ± 0.08°C in all the positive template reactions (Figure A3-2).

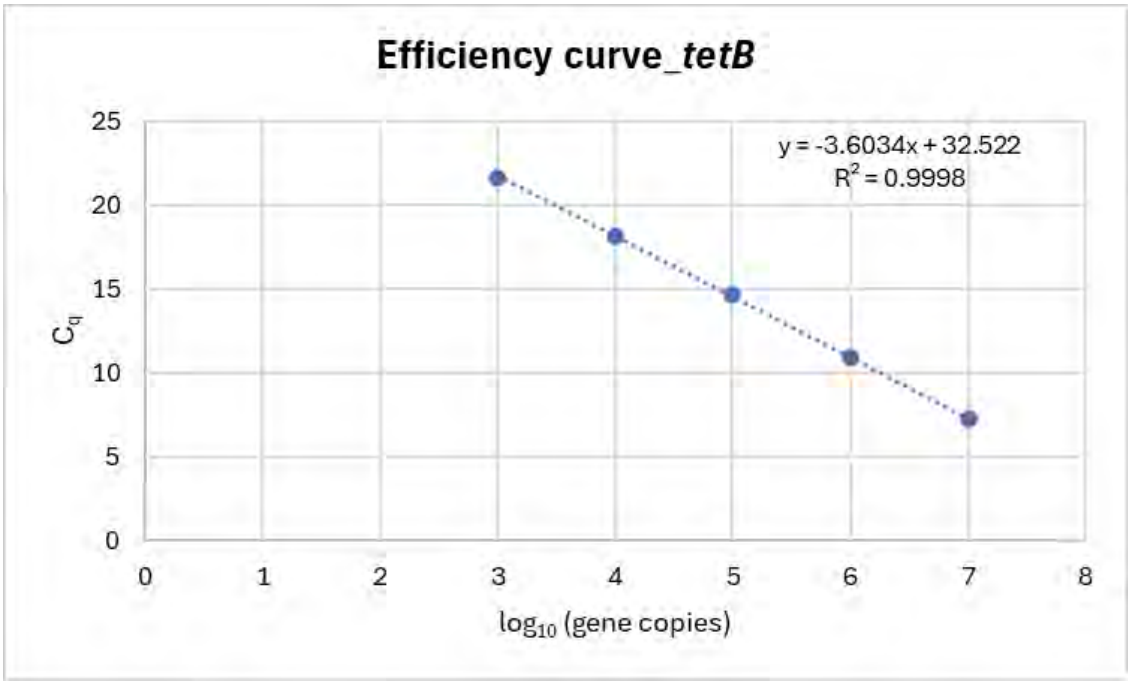

Figure A3-1. Amplification efficiency curve for the *tetB* primer. The $\log_{10}$ gene copy number is plotted against the average C_q value of the three replicates for each concentration. The standard curve equation and R^2 values are also displayed in the plot.

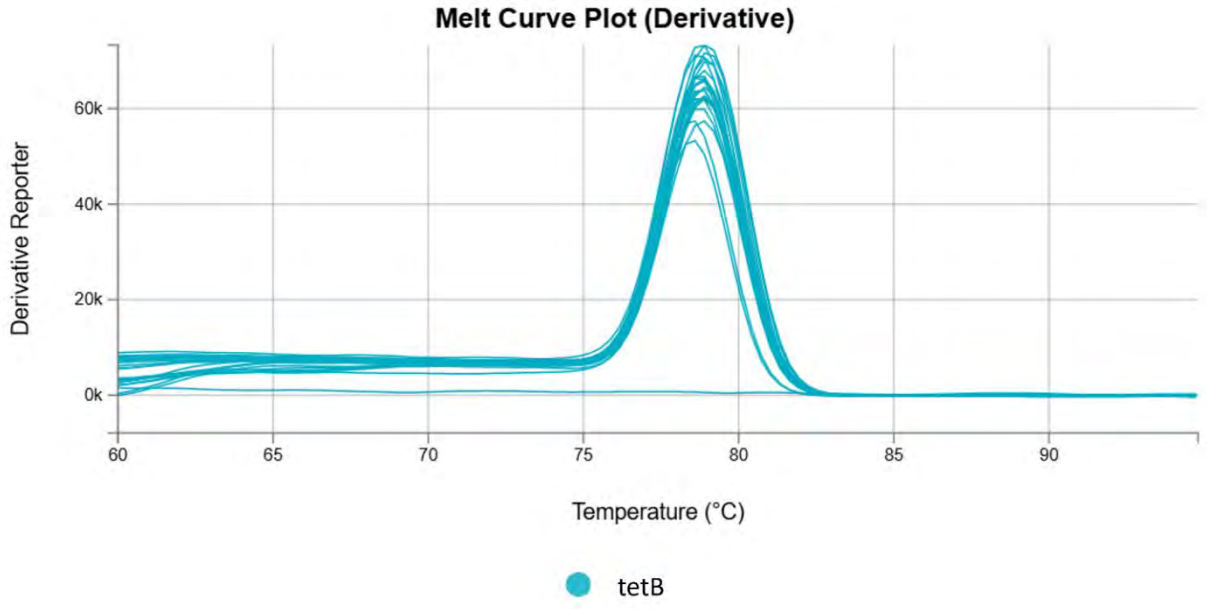

Figure A3-2. Melt curve plot for the *tetB* primer. The temperature is plotted against the negative derivative of the fluorescence divided by the derivative of the temperature. The peaks around 78ºC indicate positive amplification of the template DNA, with concentrations ranging from 10^3 to 10^7. No amplification was observed in the NTC.

- Endpoint PCR detection of *tetB* from water and microplastic biofilm samples using the *tetB* primer

The protocol for PCR detection of *tetB* was previously described in Chapter 5, Section 5.2.1. Here, an instance of endpoint PCR detection of *tetB* from water and microplastic-biofilm samples is presented (Figure A3-3).

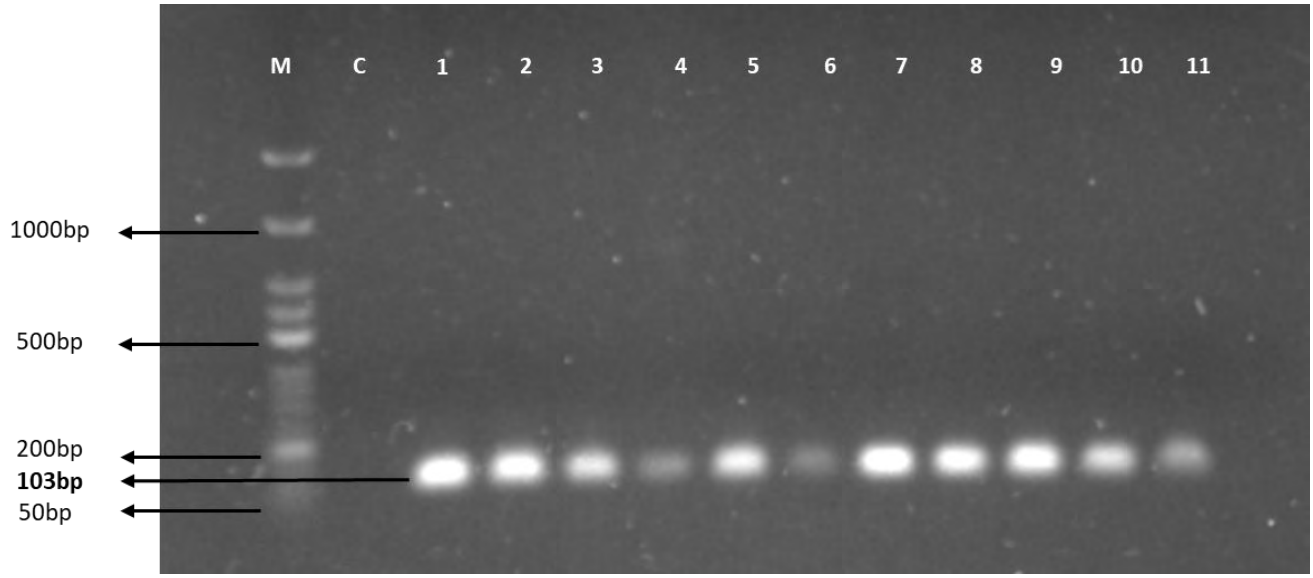

Figure A3-3. A 1% ethidium-bromide-stained agarose gels showing the detection of *tetB* from water and microplastic biofilm samples. M -– 50 bp DNA Ladder (Accuris™ Smartcheck™, USA), C – No template/negative control, *Lanes 1–11* - Positive amplification of the *tetB* gene from water and microplastic biofilm samples showing a 103 bp band.

- Amplification efficiency testing

The universal primer 515F/806R targeting the V4 hypervariable region of the bacterial *16S rRNA* gene was used as a reference gene for normalisation of *Vibrio 16S rRNA* and *tetB* gene abundances via qPCR. The primer efficiency was assessed using the same protocol as for *tetB*, described in Appendix 3. A plot of the C_q values versus the logarithm of the gene copy number resulted in a regression coefficient (R^2) of 0.998, with a slope of -3.59, corresponding to an efficiency of 90% (Figure A4-1). Melt curve analysis showed a single peak around 87.48 ± 0.12°C in all the positive template reactions (Figure A4-2).

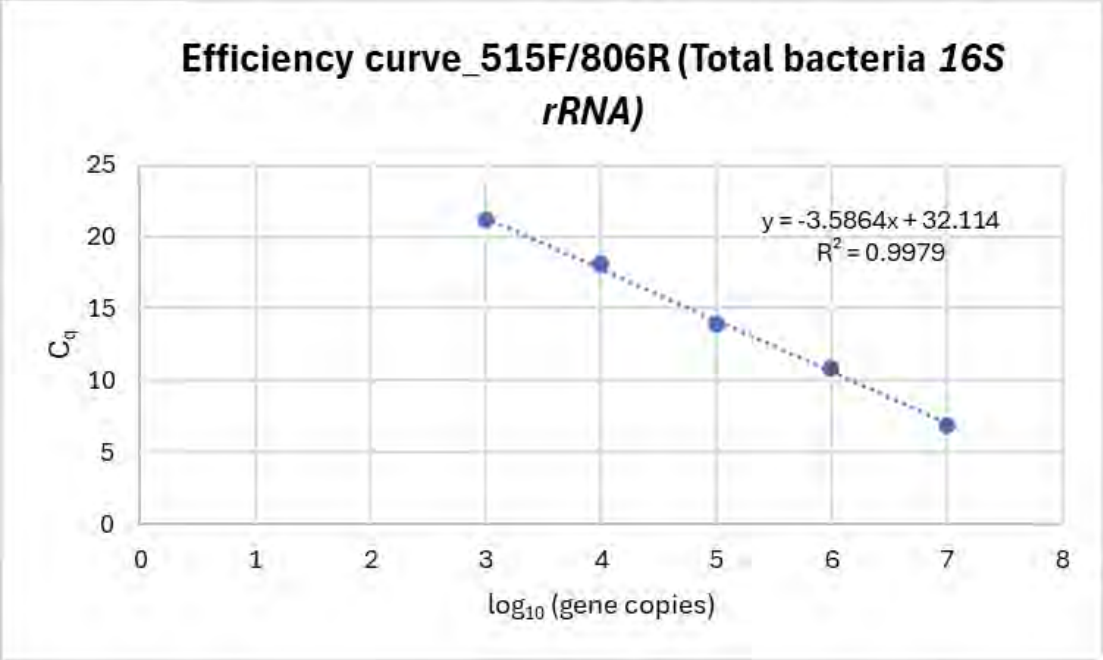

Figure A4-1. Amplification efficiency curve for the 515F/806R primer. The $\log_{10}$ gene copy number is plotted against the average C_q value of the three replicates for each concentration. The standard curve equation and R^2 values are also displayed in the plot.

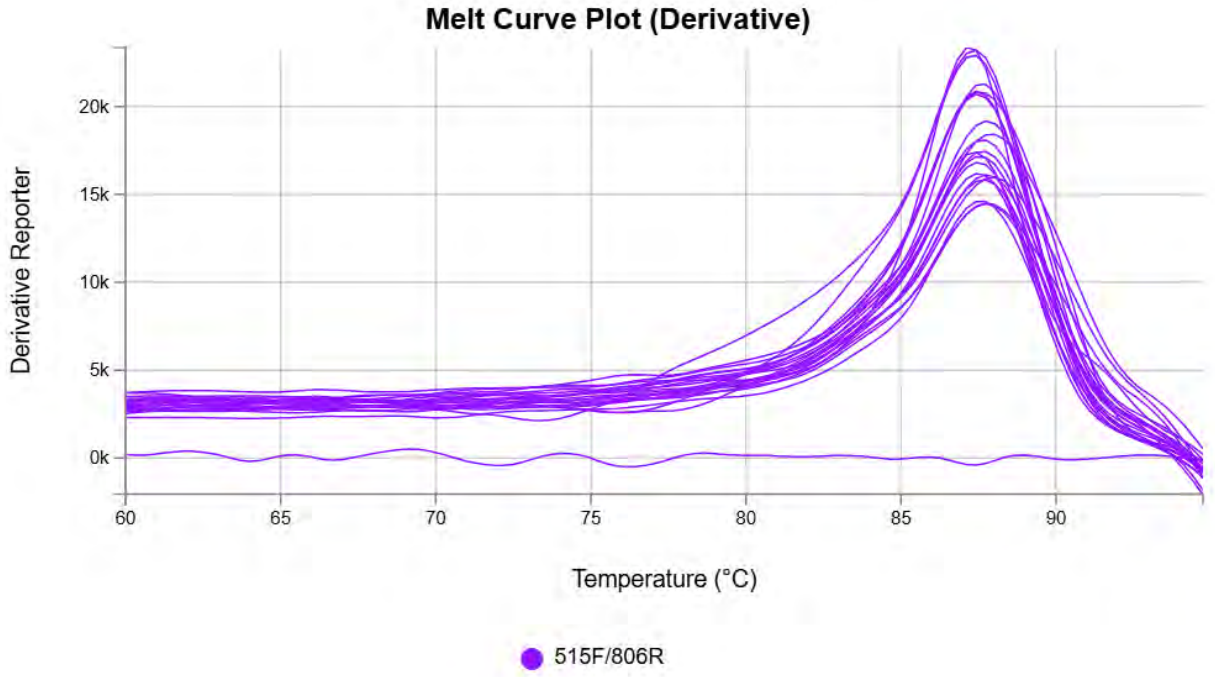

Figure A4-2. Melt curve plot for the 515F/806R primer. The temperature is plotted against the negative derivative of the fluorescence divided by the derivative of the temperature. he peaks around 87ºC indicate positive amplification of the template DNA, with concentrations ranging from 10^3 to 10^7. No amplification was observed in the NTC.

- Endpoint PCR detection of *tetB* from water and microplastic biofilm samples using the *tetB* primer

To avoid false negatives, PCR amplification was conducted to detect the presence of bacteria in the DNA samples extracted from microplastics and water samples from the Kat and Swartkops rivers. The protocol used for PCR detection of total bacteria was described in Chapter 5, Section 5.2.1. Here, an instance of endpoint PCR detection of total bacteria from water and microplastic-biofilm samples is presented (Figure A4-3).

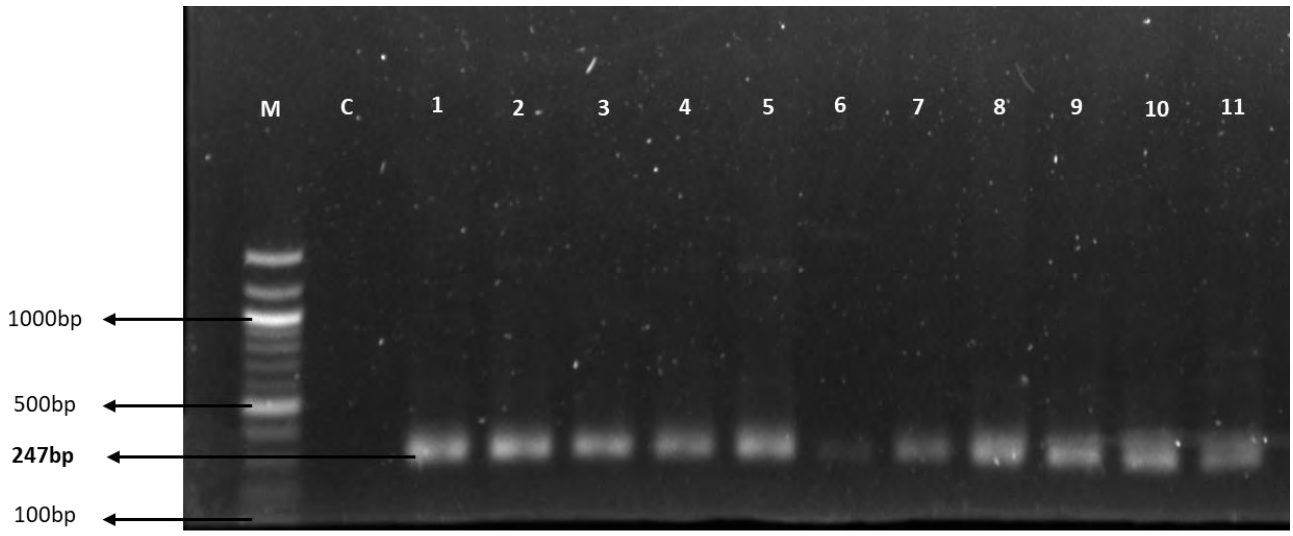

Figure A4-3. A 1% ethidium-bromide-stained agarose gel showing the detection of bacteria from water and microplastic biofilm samples. M – 100 bp DNA ladder (New England Biolabs®, USA), C – No template/negative control, *Lanes 1–11* - Positive amplification of the bacterial *16S rRNA* gene from water and microplastic biofilm samples showing a 247 bp band.

Appendix 5: Manuscripts developed from this thesis and their publication status

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Two manuscripts have been developed from this thesis and are currently at various stages of peer review. The first manuscript developed, based on Chapter 4, is titled “Design and validation of a novel primer targeting *recA* for the detection of *Vibrio* spp. in environmental water samples”. This manuscript was submitted to *Environmental DNA* on 16 January 2025 and is currently undergoing peer review (Figure A5-1). The second manuscript developed from this thesis was based on Chapter 5 and is titled “Quantification of *Vibrio* spp. and *tetB* in Water and Microplastic Biofilms in Urban Rivers in Eastern Cape, South Africa: Environmental and Public Health Implications”. This manuscript was submitted to *Scientific Reports* on 31 January 2025. The first response by the editor has been received, indicating only minor revisions to the manuscript and is currently under peer review (Figure A5-2).

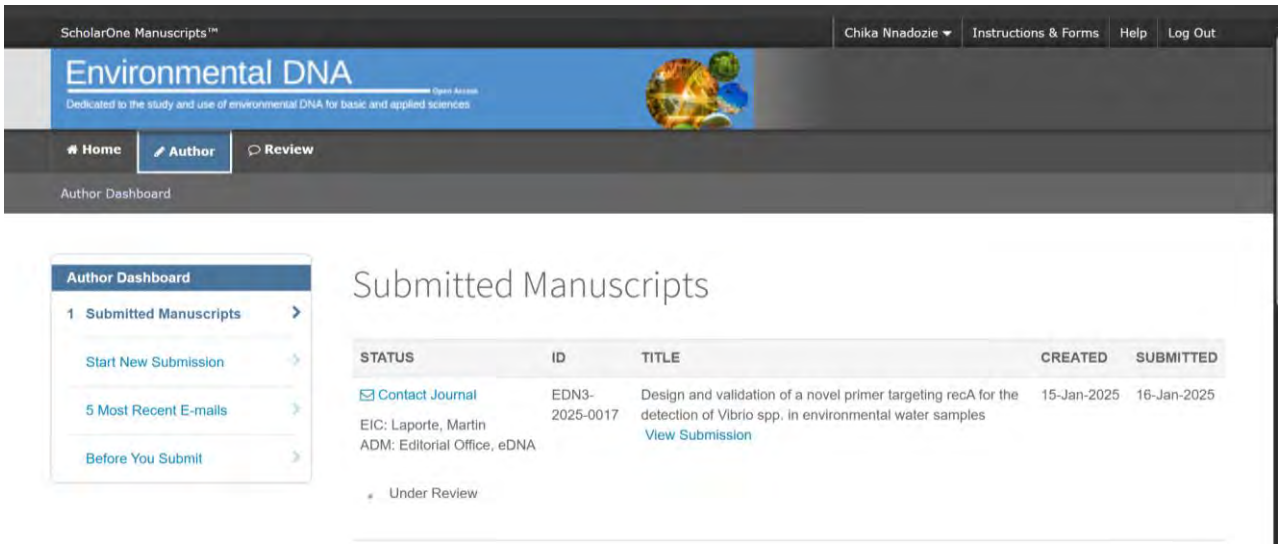
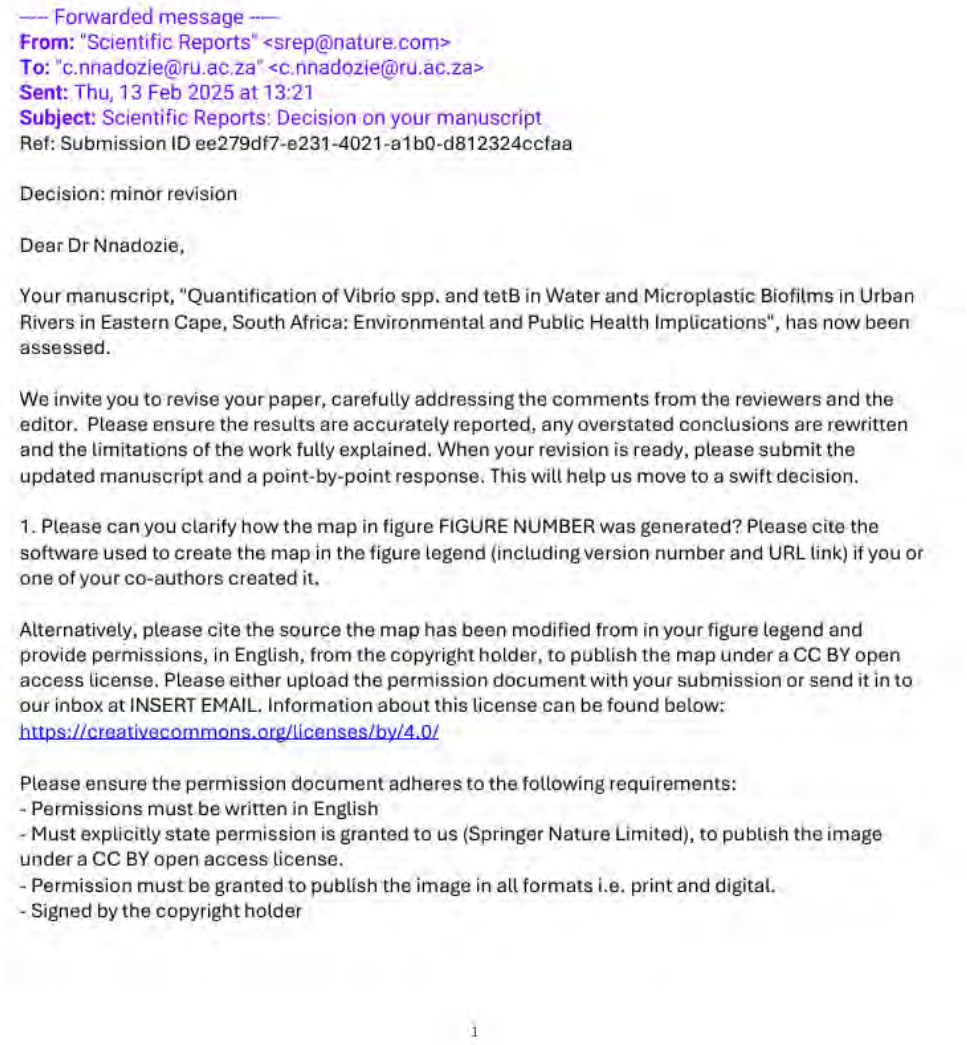


Figure A5-1. Screenshot of the *Environmental DNA* author gateway showing the submission details and peer review status of the manuscript titled “Design and validation of a novel primer targeting *recA* for the detection of *Vibrio* spp. in environmental water samples”



---- Forwarded message ----
From: "Scientific Reports" <srep@nature.com>
To: "c.nnadozie@ru.ac.za" <c.nnadozie@ru.ac.za>
Sent: Thu, 13 Feb 2025 at 13:21
Subject: Scientific Reports: Decision on your manuscript
Ref: Submission ID ee279df7-e231-4021-a1b0-d812324ccfaa

Decision: minor revision

Dear Dr Nnadozie,

Your manuscript, "Quantification of Vibrio spp. and tetB in Water and Microplastic Biofilms in Urban Rivers in Eastern Cape, South Africa: Environmental and Public Health Implications", has now been assessed.

We invite you to revise your paper, carefully addressing the comments from the reviewers and the editor. Please ensure the results are accurately reported, any overstated conclusions are rewritten and the limitations of the work fully explained. When your revision is ready, please submit the updated manuscript and a point-by-point response. This will help us move to a swift decision.

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Figure A5-2. Screenshot of an email from the editor in charge of the manuscript titled "Quantification of *Vibrio* spp. and *tetB* in Water and Microplastic Biofilms in Urban Rivers in Eastern Cape, South Africa: Environmental and Public Health Implications" showing the editorial decision for minor revisions to the manuscript.