Characteristics of bacteria associated on biofilms in freshwater microcosms

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DECLARATION

I declare that the dissertation I submitted for the degree Masters in Environmental Sciences at the North-West University (Potchefstroom Campus), Potchefstroom, North-West, South Africa, is my independent work and has not previously been submitted by me at another university.

Signed in Potchefstroom, South Africa

Signature:

Date: November 2024

Aneli van den Heever

DEDICATION

I dedicate this work to my loving family; I never would have made it without you.

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ABSTRACT

Freshwater pollution is an ever-growing concern, due to the increasing population growth combined with a rapidly decreasing natural resource capacity, such as water. The possibility of significant problems arising from water scarcity can lead to negative consequences for the environment and its inhabitants. One of the biggest water pollution concerns is microplastics. Microplastics can serve as vectors for potentially harmful bacterial groups and reservoirs for antibiotic-resistance genes. An increase in the water pollution rate, coupled with the overused and improper use of antibiotics, contributes towards the surge of antibiotic-resistant bacteria within the environment. In light of these concerns, this study aimed to determine the characteristics of bacteria associated with biofilms surrounding microplastics in a simulated freshwater environment. The first objective was to examine the bacterial colonisation on microplastics and wood substrates through scanning electron microscopy in microcosm experiments involving river water and wastewater treatment plant effluent obtained from the North West Province in South Africa. The second objective was to determine the characteristics of the bacterial isolates throughout the incubation period. The third objective was to perform Sanger sequencing of these purified isolates to aid in the identification of the bacteria found in biofilms surrounding the microplastics and wood substrate. The fourth objective was to perform next-generation sequencing targeting the 16S rRNA bacterial genes in order to characterise the bacterial community composition (BCC) within the plastisphere post-incubation. The microcosm study revealed that bacterial colonisation of various substrates occurred within 72 hours after incubation. Scanning electron microscopy images indicated that biofilm development happened within a 28-day period, revealing diverse microbial communities on the different substrates—the surface properties combined with the increased nutrient load assisted with the microbial attachment more readily. Scanning electron microscopy images revealed that the wood particles had a higher degree of colonisation than the microplastic particles due to the surface properties and organic composition. Among the 877 pure bacterial isolates tested for various extracellular enzymes, haemolysis was the most prevalent compared to DNase, resulting in 51 isolates used for further identification. Virulence testing revealed varying levels of antibiotic resistance across the 51 microbial isolates, with 16 presenting pathogenic potential. The 16 potentially pathogenic isolates underwent Sanger sequencing, and the phylogenetic relationship was determined, with a focus on eight bacterial species. *Aeromonas sp.* and *Stenotrophomonas koreensis* were among the potentially pathogenic bacteria detected. The microbial composition of the source water and plastisphere were ascertained and compared. The source water showed variation in species composition but a reduced species richness, while the plastisphere exhibited higher species richness and diversity. Next-generation sequencing revealed that Hyphomonadaceae was the dominant

family within the plastisphere, belonging to the order Caulobacterales, within the Alphaproteobacteria class and Proteobacteria phylum.

This study confirmed the presence of pathogenic bacteria and a significant bacterial composition on microplastics, highlighting their role as vectors for pathogens and the transfer of antibiotic-resistance genes within freshwater environments. Furthermore, the ongoing discharge of inadequately treated wastewater and the persistence of plastic particles can introduce waste-related pathogens into microplastic biofilms, thereby posing a significant concern for future generations. These findings highlight the urgent need for improved wastewater treatment practices and effective plastic waste management to protect freshwater ecosystems and public health.

Keywords: Biofilm, Sanger Sequencing, Microplastics, Microcosm, Bacterial community composition, Plastisphere.

LIST OF ABBREVIATIONS

A10	Amoxicillin
ACE	Abundance-based coverage estimator
AHL	Acyl homoserine lactones
ARB	Antibiotic-resistant bacteria
ARG	Antibiotic-resistance genes
AST	Antibiotic susceptibility testing
BCC	Bacterial community composition
C30	Chloramphenicol
CLASI_FISH	Combinatorial labelling and spectral imaging–fluorescence *in-situ* hybridization
CLSI	Clinical and Laboratory Standards Institute
CLSM	Confocal Laser Scanning Microscopy
CS	Constant sequences
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
eDNA	Environmental DNA
EPS	Extracellular Polymeric Substance
FISH	Fluorescence *in-situ* hybridization
HCl	Hydrochloric acid
HDPE	High-density polyethylene
HDPS	High-density polystyrene
HDSM	Hexamethyldisilane
HGT	Horizontal gene transfer
IS	Insertion sequences
ISCR	Insertion Sequence Common Region

IUPAC	International Union of Pure and Applied Chemistry
K30	Kanamycin
LDPE	Low-density polyethylene
MGE	Mobile genetic elements
NETs	Neutrophil extracellular traps
NGS	Next-generation sequencing
nMDS	Non-metric multidimensional scaling
NWP	North West Province
NWU	North-West University
OTU	Operational Taxonomy Units
PAH	Polycyclic aromatic hydrocarbons
PBDE	Polychlorinated diphenyl ethers
PCB	Polychlorinated biphenyls
PCR	Polymerase chain reaction
PET	Polyethylene terephthalate
POP	Persistent organic pollutant
PP	Polypropylene
PPI	Pathogenic potential index
PVC	Polyvinylchloride
PS	Polystyrene
PUR	Polyurethane
RP	Residence plasmid
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
R2A	Reasoner’s 2A Agar

RO	Reverse osmosis
SA	South Africa
s.d.	standard deviation
SEM	Scanning electron microscopy
SMP	Synaptotagmin-like mitochondrial-lipid-binding
T5	Trimethoprim
VA30	Vancomycin
WWTP	Wastewater treatment plant
UV	ultra-violet

LIST OF NOMENCLATURE

bp	Base pair
°C	Degree Celsius
cm	Centimetre
g/cm^3	Gram per cubic centimetre
Kb	Kilobase
kt	Kiloton
KOW	Octanol-water partition coefficient
kV	Kilovolt
M	Mol
mm	Millimetre
m^3	Cubic metre
ng	nanogram
pmol	Picomoles per Liter
V	Volt
µg	Microgram
µl	Microlitre
µM	Micrometre

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Chapter 1 : Introduction

1.1 General Introduction

Water scarcity is an increasingly critical issue compounded by elevated pollution risks and population growth (Dallas & Rivers-Moore, 2014). In South Africa, the exacerbation of water scarcity can be attributed to a combination of environmental pressures and inadequate management practices (Dallas & Rivers-Moore, 2014; Hedden & Cilliers, 2014). The freshwater environment consists of different nutrients derived from the surrounding environment, along with low concentrations of dissolved salts, dissolved solids, as well as organic and inorganic matter (Cole *et al*., 2011). The influx of these nutrients and contaminants derived from anthropogenic activities further compounds the risk of freshwater pollution within a water-scarce South Africa (Mvovo, 2021).

Plastic pollution has rapidly become one of the most pervasive environmental issues, impacting both terrestrial and aquatic environments due to the possible impacts on the organisms within that environment (Azevedo-Santos *et al*., 2021; Mvovo, 2021). This problematic rise is primarily attributed to the surge in plastic production and consumption, coupled with inadequate waste disposal practices (Rummel *et al*., 2017). Microplastics are seen as a significant pollution source among the various forms of plastic pollution due to their shape, size and abundance within the environment (Mvovo, 2021). Microplastics (<5 mm) are either primary or secondary, depending on the source requirements and fragmentation capabilities (Eriksen, 2014; Lambert & Wagner, 2018). It has been revealed that microplastics are amassing in oceans and inland waters and hindering aquatic biodiversity (Mvovo, 2021). These polymer fragments can also be misinterpreted as food sources by aquatic life (Lee *et al*., 2013; Naidoo *et al*., 2020).

1.2. Microplastics and the aquatic environment

The introduction of microplastics into the aquatic environments is exacerbated by various anthropogenic activities, such as industrial discharges, sewage outfalls, and agricultural runoff (Avio *et al*., 2016; Blair Espinoza, 2019). Environmental phenomena such as flooding, hurricanes and extreme rain can further increase microplastic pollution within the water systems (Cole *et al*., 2011). Moreover, these plastic particles may carry organic contaminants, either absorbed from the surrounding environment or added during plastic production (Blair Espinoza, 2019; Pareao *et al*., 2020).

One of the biggest threats towards South African freshwater systems is inadequately functioning wastewater treatment plants (Oharisi *et al*., 2023). Wastewater treatment plants function as the frontline for eliminating large plastic particles, reducing nutrients and organic compound concentrations before discharging the effluent into the environment (Magnusson & Norén, 2014). However, these wastewater treatment plants can also serve as an introductory source for microplastics, pathogenic bacteria, antibiotic-resistant bacteria, and contaminants within the aquatic environment (Talbot & Chang, 2022; Makuwa *et al*., 2022). The release of wastewater effluent into the environment can alter the environmental composition with regard to the pre-existing and newly introduced microbes (Donian, 2002).

The extensive use of antibiotics in human and veterinary medicine, combined with the increased use for agricultural needs, results in the increased prevalence of antibiotic-resistance genes and antibiotic-resistant bacteria within the aquatic environment, which can be attributed to the partially metabolised antibiotics entering the wastewater treatment plants (Li *et al*., 2014; Oharisi *et al*., 2023). These antibiotic-resistant bacteria contain antibiotic-resistance genes, which can proliferate through horizontal gene transfer mechanisms and disseminate into the aquatic environment and, in doing so, distribute the antibiotic-resistance genes to various bacteria and ultimately pose a threat to human health (Li *et al*., 2014; Phungela *et al*.,2022).

Biofilm formation within freshwater systems can be influenced by environmental signals, such as nutrients and pollutants (Rodriguez *et al*., 2015). These environmental signals can result in an increased multicellular bacterial attachment on the microplastic particles that were present in the wastewater treatment plant effluent (Mah, 2012; Rodriguez ., 2015). These biofilms differ from their free-floating counterparts in terms of gene expression and the retention of harmful genes through horizontal gene expression (Donian, 2002). Microplastics are, therefore, seen as ‘hotspots’ for pathogenic bacteria within the aquatic environment.

1.2. Problem statement

The escalating issue of plastic pollution, driven by increased productivity and improper disposal of plastic material, has led to a rise in plastic pollution (Cole *et al*., 2011). The water quality of natural freshwater systems in a water-scarce South Africa is under constant threat. The numerous environmental pressures, the increased population and mismanagement of resources have led to the deterioration of these freshwater systems. The ineffectiveness of wastewater treatment plants in removing smaller plastic particles and excessive nutrients results in an increased amount of microplastics, antibiotics and toxins entering the aquatic environment through various routes (Zettler *et al*., 2013). Numerous studies have reported the

presence of microbes surrounding microplastics within freshwater systems (Mah, 2012; Pareao *et al*., 2020; Iloms *et al*., 2020). However, more information regarding the characterisation of these bacteria surrounding various microplastic polymer types within freshwater environments is needed, with a focus on South African water systems.

1.3 Aim

This research on microplastics aimed to identify and determine the characteristics of bacterial isolates found within biofilms attached to microplastics within simulated freshwater environments, along with investigating the bacterial composition of the biofilms from river water and wastewater treatment plant effluent obtained from the wastewater treatment plant in Potchefstroom, North West, South Africa. Therefore, analysing the 'plastisphere' (Schlundt *et al*., 2020) would provide insight into the bacterial diversity and its role in dispersing antibiotic-resistant bacteria (ARB) and antibiotic-resistance genes (ARG) within the water column.

1.4. Objectives

The objectives include the following:

- To use scanning electron microscopy to visualize temporal biofilm formation on microplastics in river water and wastewater treatment plant effluent".
- To determine the characteristics of bacterial isolates through various extracellular enzymatic tests using differential media and Gram staining.
- To determine the antibiotic susceptibility of the microbial isolates using the Kirby Bauer disc diffusion method and to identify potentially pathogenic bacteria within the aquatic environment.
- To identify the pure microbial isolates through virulence testing and Sanger sequencing.
- To determine the microbial composition surrounding the biofilm in a simulated freshwater environment to shed light on the naturally occurring microbes found within the aquatic environment.

Chapter 2 : Literature Review

2.1. South Africa's water scarcity

South Africa (SA), characterised as a semi-arid country, receives an average rainfall of 465 mm per year, which is notably lower than the 860 mm global average (Liu *et al*., 2013; Mnguni, 2020). Ranked the 30th driest country globally, SA contends with volatile rainfall, erratic runoff, high evaporation rates, and shallow dam basins (Cole *et al.,* 2018; Hedden & Cilliers, 2014; Pitman, 2014). The rainfall can increase from below 200 mm in the western region of SA to above 1200 mm per annum in the east but remains below the global average (McBride *et al*., 2022). The escalating environmental pressures, coupled with supply security and resource pollution, threaten 60% of the country's 223 river ecosystems, 25% of them being classified as critically endangered (Hedden & Cilliers, 2014; Department of Water Affairs, 2013; Filho *et al*., 2022). With just over 1200 m^3 of fresh water available per person per year for a population of approximately 56 million, SA struggles with water scarcity and is currently classified as a water-stressed nation (Iloms *et al*., 2020; Cole *et al.,* 2018; Dallas & Rivers–Moore, 2013; Filho *et al*., 2022). Water-stressed nations are seen as nations where the water need per capita falls below 1700 m^3, whereas between 1000 and 1700 m^3 water shortages can occur and below 1000 m^3 water scarcity occurs (Filho *et al*., 2022). The water scarcity in SA can be attributed to various indicators, such as surface water availability, groundwater availability, climatological stressors, and physical needs (Filho *et al*., 2022).

2.1.1 Water challenges facing South Africa

South Africa's metropolitan areas are predominately located on the watersheds of river catchments, typically situated on elevated regions that receive copious amounts of water, thereby influencing the downstream ecosystems (Oberholster & Ashton, 2008; Tzanakakis *et al*., 2020). However, the quality of the water resources has been deteriorating for some time due to the escalating industrial growth, urbanisation, afforestation, mining, agriculture, and power generation activities within these areas (Oberholster & Ashton, 2008; Li *et al*., 2017; Filho *et al*., 2022). The drainage of these rivers from the watersheds has the dual purpose of supplying water and transporting waste material, leading to the downstream accumulation of pollutants within the water storage reservoirs (Oberholster & Ashton, 2008). According to Mnguni (2020) and Filho *et al*. (2022), the South African Human Rights Commission reported that 11% of formal and informal households still require sanitation services resulting in the improper disposal of waste into nearby rivers and streams.

Unfortunately, a large portion of sewage in South African urban areas is left untreated, primarily due to rapid population growth and urbanisation which placed strain upon wastewater treatment plants (WWTP), resulting in the unsuccessful treatment of sewage and incomplete sewer systems (Oberholster & Ashton, 2008; Filho *et al*., 2022; Netshithothole *et al*., 2024). Despite having over 1000 water treatment facilities, the quality of the discharged treated water back into the river systems remains a concern. However, the direct reuse of this water is -minimal in terms of the treated water being released into rivers, streams, and dams (Hedden & Cilliers, 2014).

2.1.2. Aquatic pollution

An aquatic ecosystem can be defined as a water-based environment that occupies 75% of the world's surface (Sreelakshmi & Chitra, 2021). The aquatic ecosystem can be influenced by human interventions, such as agricultural practices, urbanisation, industrialisation, soil erosion, and over-exploitation, which can result in the significant degradation of these ecosystems (Sreelakshmi & Chitra, 2021). Aquatic pollution can, therefore, be described as the addition and ingress of substrates and materials into water bodies which are not a normal part of the water cycle (Qadri & Faiq, 2020). Human-made pollution sources include industrial and chemical wastes discharged into water bodies due to accidental or intentional spillages and the dumping of wastewater and untreated effluents (Sreelakshmi & Chitra, 2021). Moreover, improper waste disposal practices can lead to the influx of excessive nutrients and minerals, thereby influencing microbial growth and abundance within the system, promoting phenomena such as excessive algal blooms (Lambert & Wagner, 2018; Ting *et al*., 2021).

2.2. Plastic pollution and consumption in South Africa

According to Vert *et al*. (2012), the International Union of Pure and Applied Chemistry (IUPAC) defined plastic as a polymeric material that can contain other substances to improve performance and reduce cost. Plastics can be separated into two main groups, namely, thermoplastics and thermosets (Blair Espinoza, 2019). Thermoplastics are solids at room temperature and can be melted, hardened, and reshaped repeatedly; these include polypropylene (PP), polystyrene (PS) polyvinylchloride (PVC), and polyethylene in comparison to thermosets, which are liquids at room temperature and are chemically altered when heated, for instance, polyurethane (PUR) (Espinoza & Maricela, 2019)

Since its development in 1907 as a phenol-formaldehyde resin, plastic has emerged as one of the most ubiquitously utilised materials, attributed to its advantageous properties such as low weight, durability, ease of production, and cost-effectiveness (Boyle & Örmeci, 2020;

Ziajahromi, 2018). An increased production rate, low recycling rate, and lack of plastic degradation resulted in the accumulation of thermoplastic within the aquatic environment (Boyle & Örmeci, 2020). Plastic pollution can, therefore, be defined as the introduction of plastics, regardless of size, shape, or type, into the environment, resulting in potential threats to the environment, organisms, and human health (Li *et al.,* 2020). According to Boyle & Örmeci (2020), approximately 90% of the total world plastic production consists of polyethylene terephthalate (PET), high-density polyethylene (HDPE), low-density polyethylene (LDPE), PVC, PP and PUR. These plastics account for an estimated 10% of solid waste and contribute approximately five trillion plastic particles within the marine environment (Ziajahromi, 2018).

The production of different polymers reaches more than 335 million tons each year; several plastic materials can be reused or recycled, but many others are discarded after use, also known as single-use plastics (Azevedo-Santos *et al.,* 2020; Li *et al.,* 2020). Therefore, environmental plastic pollution can be categorised according to the plastic characteristics (Size, shape, type, colour, and sources), degradation resistance, combined pollutants, and potential risks (Li *et al.,* 2020; Cai *et al.,* 2018). The diversity of plastic pollution stems from various sources, anthropogenic activities, the density of the plastics, and the numerous variations (Li *et al.,* 2020; Singh & Devi, 2019). Additionally, plastic pollution manifests differently across various ecosystems; the migration of plastics within these systems leads to the accumulation of multiple types of plastics within the environment (Li *et al.,* 2020).

The lack of control over the production and disposal chains of plastic leads to incorrect disposal, thereby ultimately entering and altering the aquatic environment through various routes (Azevedo-Santos *et al.,* 2020; Li *et al*., 2020). Plastic can also join the aquatic environment through environmental changes, such as hurricanes, flooding, and severe rain (Cole *et al.,* 2011). The density of these plastic particles influences the geographical location within a specific environment as well as the general area; plastics with a lower density than the surrounding freshwater (approx. 1.0 g/cm^3) are buoyant compared to those with higher density, thereby being more likely transported to different environments (Duis & Coors, 2016).

According to Azevedo-Santos *et al.* (2020), plastic pollution has numerous consequences for freshwater biodiversity, environments, and ecosystems. Plastic can cause physical, toxic, and behavioural impacts, but its association with other pollutants may enhance its effects. The freshwater biota can be affected by plastic pollution through physical interactions. Plastic pollution is considered a crucial environmental problem and an emerging issue that might affect human health and diversity in the near- to medium-term future (Blettler *et al.,* 2017).

In SA, approximately 37% of households do not have access to weekly waste collection, leaving 29% of all household waste uncollected and improperly disposed of (Stafford *et al.,* 2022), resulting in plastic accumulation within the environment. According to Stafford *et al.* (2022), the current annual plastic waste generated is 1546 kt (Kiloton), of which only 1350 kt is collected. The majority of the collected plastic waste is collected in the form of mixed municipal solid waste, with little to no separation, but 301 kt is recycled per annum. As much as 488 kt enters the environment due to mismanagement and improper disposal, where it contributes to air pollution through open-air burning (275 kt), land pollution (145 kt) and aquatic pollution (68 kt) (Stafford *et al.,* 2022). When contrasted against air and land pollution, aquatic pollution is a relatively small component of SA's pollution problems, but it is an increasing threat to the aquatic ecosystems in and surrounding SA, thereby posing a threat towards the freshwater systems.

2.3. Plastic and the aquatic food web

Plastic disintegration mechanisms within the environment can either be chemical or physical (Plohl *et al*., 2022). Physical degradation appears in structural changes, such as cracking, embrittlement, fragmentation and flaking, while chemical degradation is related to changes at molecular levels, such as bond cleavage, hydrolysis and oxidation of polymer chains (Plohl *et al*., 2022; Li *et al.,* 2020; Cai *et al.,* 2018). Plastic disintegration within aquatic environments is facilitated by increased temperatures, water turbulences, ingestion, and the activities of organisms like isopods (Duis & Coors, 2016; Bank & Hansson, 2019).

The disintegration of common polymers, such as LDPE, HDPE and PP, is initiated by UV (ultra-violet) radiation, which leads to the plastics becoming brittle and fragmenting, resulting in the possible predation (Duis & Coors, 2016; Weideman *et al*., 2020). Plastic disintegration is, therefore, more likely to infiltrate the aquatic food web (Mvovo, 2021; Sheridan *et al*., 2022). Aquatic alga or plants may absorb small-sized plastic particles, which are then ingested by the invertebrates. Numerous invertebrate species are frequently exposed to the accumulated plastic particles within the sediment (Azevedo-Santos *et al.,* 2020). Therefore, invertebrates are seen as an important input into the aquatic food web.

Microplastics are seen to have a widespread distribution and motility, but have a similar smaller size compared to prey organisms, resulting in the ingestion by these invertebrates (Ory *et al.,* 2017). Fish can ingest microplastics in two scenarios: by feeding on the microplastic-infested invertebrates or through direct ingestion of these particles due to the density, movement, colour, and shape, which can imitate the micro-invertebrates (Wright *et al.,* 2013). Once ingested by fish, these plastic particles can cause intestinal injuries, gut

distension, organ failure, and abnormal swimming behaviour and may lead to secondary effects, such as increased vulnerability to predation due to the increased weight associated with microplastic ingestion (Azevedo-Santos *et al.,* 2020; Naidoo *et al.,* 2020). Amphibians can either prey on fish or ingest these free-floating plastic particles. Reptiles are predatory in nature and, therefore, feed on amphibians or fish. Thereby ingesting plastic directly or indirectly. Birds and mammals can feed on the fish or drink the water containing the free-floating plastic particles as well (Azevedo-Santos *et al.,* 2020). Birds may ingest plastic particles directly or indirectly, which may lead to digestive tract obstructions, increased risk of starvation, and predation.

Lee *et al.* (2013) investigated the uptake of both nano- and micro-sized PS spheres by the marine copepod, *Trigriopus japonicus.* The spheres were detected within the gut of the copepod after 24 hours, indicating predation on the part of the copepod and the accumulation of plastic particles in its digestive tract (Lee *et al.,* 2013). Cousin *et al.* (2020) indicated that PS microparticles were ingested by freshwater protozoans, *Paramecium* and *Daphnia* species where, after ingestion, the plastic particulates can either be excreted, remain within the digestive tract, or be absorbed into the body tissue (Duis & Coors, 2016). Setälä *et al.* (2014) observed the trophic transfer of fluorescent PS microspheres from zooplankton to the mysid shrimp, *Mysis relicta,* while Boerger *et al.* (2010) investigated the plastic concentration in the gut of small pelagic fish, with approximately 35% of the fish having plastic particles in their stomach, and 94% of the ingested plastics existing in fragments. Sanchez *et al.* (2014) found that in wild gudgeons (*Gobio gobio*), the digestive tracts contained microplastics, which can be attributed to the increased anthropogenic activities surrounding the streams. Plastic particles, including microplastics, have also been shown to be ingested by both birds and mammals (Azevedo-Santos *et al*., 2021; Duis & Coors, 2016).

Cox *et al.* (2019) evaluated the human consumption of microplastics in adults to find that ingestion through drinking, salts, bivalves and fish, as well as the inhalation of plastic-contained air and skin occurred. Lehner *et al.* (2019) meanwhile reported that microplastics can enter human cells, causing oxidative stress and inflammation within the cells, leading to cell disintegration. Freshwater systems, therefore, act as reservoirs for microplastics, posing the risk of possible ingestion by humans.

2.4. Microplastic life cycle

According to Duis and Coors (2016) and Day *et al.* (2022), microplastics can be defined as synthetic organic polymer matrix or solid particles with a regular or irregular shape, insoluble, smaller than 5mm, and from either primary or secondary manufacturing origin. Primary

microplastics are defined as microplastics produced in a micro size range, compared to secondary microplastics, which result from more extensive plastic fragmentation and degradation (Duis & Coors, 2016; Li *et al*., 2020).

Primary microplastics are used in a variety of different ways, such as exfoliants in personal care products, such as sanitisers, facial cleaners, and various kinds of toothpaste, in medical applications, and as carriers to deliver pharmaceutical agents (Avio *et al*., 2016; Hale *et al*., 2020). Industrial abrasives, and plastic products fabrication through raw materials are also seen as primary microplastics (Duis & Coors, 2016). The raw materials can enter the aquatic environment through accidental loss or runoff from processing facilities due to improper handling (Avio *et al*., 2016; Duis & Coors, 2016) (Figure 2.1).

Secondary microplastics are due to the degradation and fragmentation of larger plastics that enter the aquatic environment through the dumping of plastic waste and loss from inappropriately managed landfill sites and during waste collection (Hale *et al*., 2020; Duis & Coors, 2016). Low-density polyethylene is used in large volumes to aid in crop production, weed suppression and irrigation water retention and, therefore, there is a higher chance of these particles entering the aquatic environment (Duis & Coors, 2016). Various sources of microplastics also include synthetic textiles, protective paints, manufacturing and construction emissions as well as car tyre abrasions (Lambert & Wagner, 2018) (Figure 2.1).

Factors that can influence microplastic degradation include environmental exposure conditions and polymer properties such as the crystallinity, quantity, and quality of the chemical additives (Lambert & Wagner, 2018; Ivar do Sul & Costa, 2013). The crystallinity of a polymer is described as the crystalline region that consists of ordered and tightly structured polymer chains, which influences the permeability and density of the polymer, resulting in the position of the plastic particle within the water column (Lambert & Wagner, 2018). This, in turn, affects the hydration and swelling of the particle and, in doing so, affects the accessibility of sorption sites of microorganisms (Lambert & Wagner, 2018).

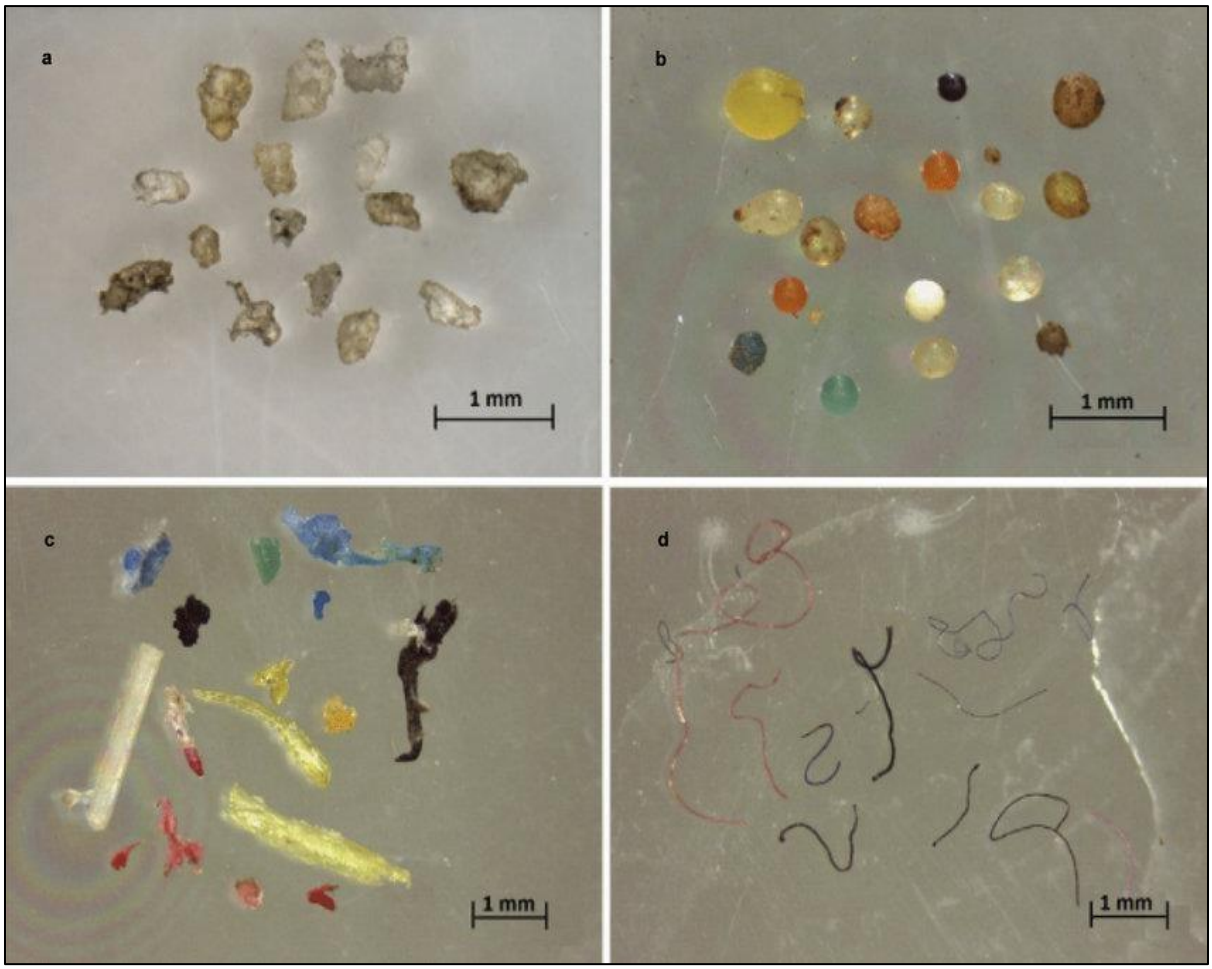


Figure 2.1: Images showing the difference between the two groups of microplastics: primary microplastics derived from personal care products (a and b) and secondary microplastics derived from the breakdown of larger plastic fragments and synthetic fibres (c and d) (adapted from Koyuncuoğlu & Erden, 2021).

As stated, smaller plastic fragments are more readily ingested by aquatic animals; thereby, the microplastic ingestion probability is higher compared to the larger plastic particles (Wagner *et al.,* 2014). Microplastics possess the ability to accumulate heavy metals such as cadmium (Cd), gold (Au), copper (Cu), iron (Fe), zinc (Zn) and lead (Pb). Polyvinylchloride can accumulate these heavy metals and increase the metal concentration up to 800 times (Xu *et al.,* 2019). By concentrating these heavy metals that are produced and released by industrialisation, microplastics can alter their toxicity and heavy metal localisation, depending on size, shape, and the substrate coating layer (Xu *et al.,* 2019). Due to their specific shape and size, microplastics exhibit higher concentrations of organic and inorganic chemicals, as well as heavy metals, compared to the surrounding aquatic environment, suggesting that microplastics have a higher affinity towards these chemicals (Avio *et al.,* 2016; Wagner *et al.,* 2014). This affinity can be attributed to the chemical, physical and biological factors that allow the chemical concentrations to increase over time through the absorption of particles and

accumulation by biofilms forming around microplastics (Avio *et al.,* 2016). Therefore, the attachment of microorganisms and absorption of chemicals to organic and inorganic particles in aquatic environments can be attributed to the ecosystem, temporal changes, and spatial changes (Galgani *et al.,* 2018). Upon ingestion of microplastics, these compounds can transfer to the organisms and act as vectors for organic pollutants and, therefore, increase chemical exposure to aquatic wildlife (Wagner *et al.,* 2014; Li *et al.,* 2020).

2.5. Microplastics in freshwater environments

In the freshwater environment, the amount of microplastics released into the rivers or streams is dependent on the population density, proximity to urban areas, the layout of the environment, WWTP and the continuous fragmentation of macro-plastic particles (Ziajahromi, 2018; Xu *et al.,* 2019). Freshwater aquatic environments are naturally located in valleys and lower-elevation terrains; therefore, the incorrect disposal of plastics can be carried by pluvial waters to waterbodies along with direct release into freshwater bodies (Azevedo-Santos *et al.,* 2021). According to Talbot and Chang (2022), the amount of microplastics released into rivers and tributaries is comparable to river mainstems and larger water bodies, such as lakes. According to Deya *et al.* (2020), 70 – 80% of microplastic waste is transported through river systems into the oceans.

According to Wagner *et al.* (2014), major contributors to freshwater microplastic pollution include WWTP effluent, urban, industrial, touristic, and agricultural runoff, as well as sludge that contains microplastics, due to the ineffectiveness of the WWTP to remove such particles. Sewage sludge is continuously used as a fertilising agent for agriculture and landfilling; thus, surface runoff can lead to the transfer of microplastics into freshwater environments (Wagner *et al.,* 2014; Weideman *et al*., 2019; Weideman *et al*., 2020). Microplastics in freshwater environments are of great concern due to their potential to bioaccumulate toxins and heavy metals, which increases with a decrease in size (Wagner *et al.*, 2014).

According to Lambert and Wagner (2018), the movement of these particles within riverine systems is also determined by the hydrology and morphology of a specific river, which can influence the accumulation of deposition of these particles. Physical and chemical weathering fragment synthetic polymers into smaller particles over time, increasing the number of plastic particles in the freshwater ecosystems. Thus, improper waste management, durability and unsustainable plastic use gave rise to extensive accumulation of plastics in the aquatic environment (Wagner *et al.,* 2014).

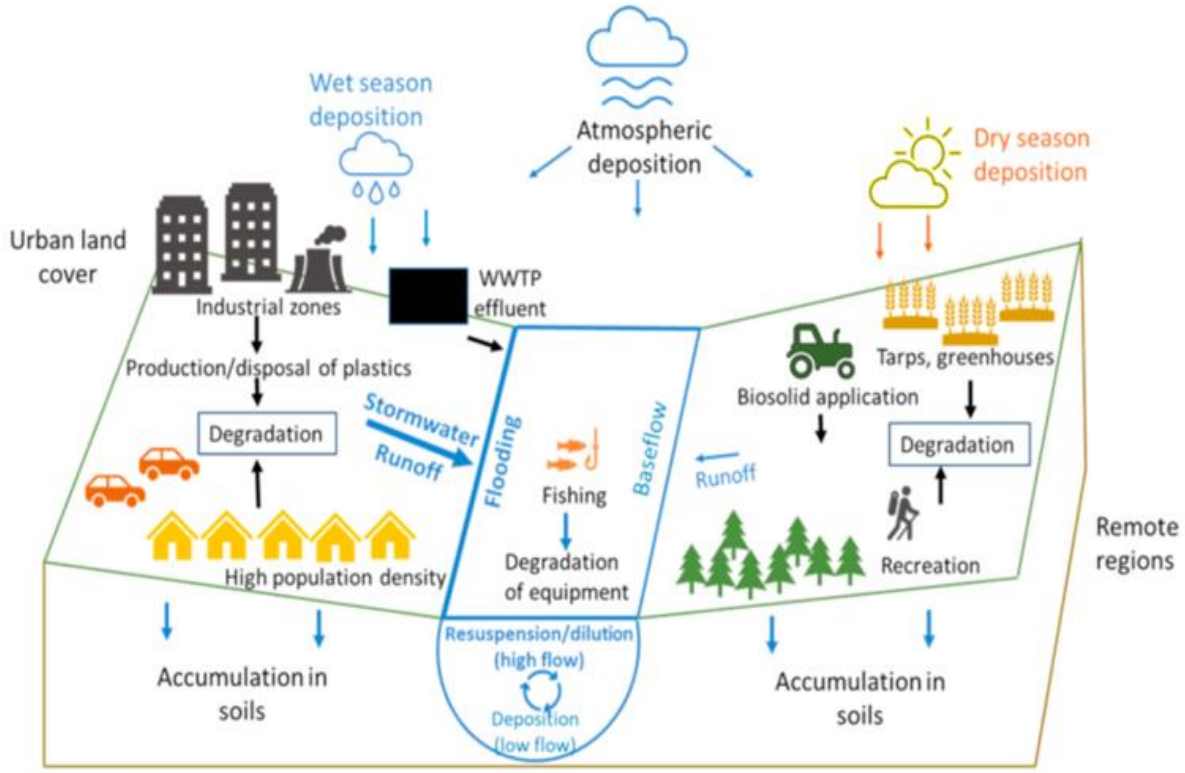


Figure 2.2: Conceptual model of the spatial and temporal factors influencing the distribution and abundance of freshwater microplastics (adapted from Talbot & Chang, 2022).

Figure 2.2 above outlines these components of the microplastic cycle within river systems with a focus on anthropogenic sources and introduction within freshwater bodies. Factors that influence the temporal distribution include precipitation seasonality, storm runoff, and flow; precipitation can serve as transport for land-based microplastics into the aquatic environment. Schmidt *et al*. (2018) hypothesised that precipitation may lead to a first flush event, in which the land-accumulated microplastics are flushed into freshwater environments during the wet seasons. Microplastics are influenced by water velocity, lower flow rates and weakened hydrodynamics, facilitating the accumulation within the sediment of a river system (Talbot & Chang, 2022). As previously mentioned, spatial distribution is influenced by anthropogenic activities and watershed/stream characteristics (Talbot & Chang, 2022; Lambert & Wagner, 2018).

According to Weideman *et al*. (2020), the transportation of microplastics of different sizes within river systems is poorly known due to the civilisation surrounding the river banks, making it hard to differentiate between the microplastics introduced to the river due to improper waste disposal, or introduced upstream. Weideman *et al*. (2020), studied the transportation of microplastics within the Orange - and Vaal Rivers in SA, and found that the microplastic concentration was greatly influenced by the surrounding urbanized areas. Weideman *et al*.

(2020), found that the temporal distribution within the river system was also influenced by the seasonality, climate change and the surrounding activities.

Freshwater environments, such as lakes, rivers and reservoirs may act as sinks for microplastic contaminants due to the high residence times within these environments (Nel *et al.,* 2018). Nel *et al*. (2018), studied the role of river sediment as a repository for microplastics within the water column and found that the environmental pressures and flow dynamics within a river, the microplastic contamination within sediments, influence. The sediments of freshwater systems can therefore also act as a source for microplastics. Dahms *et al*. (2020) found that the sediment type also plays a role in the number of microplastics found within river banks, the finer sediment profiles with higher organic content allow the microplastics to become trapped and require more energy to be dislodged compared to the coarse sediment. Therefore, the flow velocity, depth and environmental changes can influence the retention time of microplastics within the water system.

2.6. Wastewater pollution: effects of emerging contaminants

Wastewater treatment plants function as the frontline to eliminate large particles and reduce nutrient and organic compound concentrations (Magnusson & Norén, 2014). Wastewater treatment plant influent is generated by industries, urban runoff, household, and commercial establishments, whilst the effluent can enter rivers, streams, lakes and oceans (Magnusson & Norén, 2014). The amount of people in an area can determine what type of WWTP is used (Talvitie *et al.,* 2015). Thus, depending on the type of WWTP, it can have a primary, secondary, and tertiary treatment that can purify the influent by using physical, chemical, and biological treatments (Ziajahromi *et al.,* 2016). The WWTP in SA consists of primary and secondary treatment phase (Weideman *et al*., 2020).

During the primary treatment step in WWTP, the course-suspended solids are removed through screens/sieves, the heavy particulates and the floating materials are removed through grease separators (Duis & Coors, 2016). The coarse screen openings are approximately between 20–50 mm, intermediate screens between 10–20 mm and fine screens between 2–10 mm (Duis & Coors, 2016). The secondary treatment stage uses biological and chemical processes to remove organic matter, whereas the tertiary treatment stage involves the removal of nutrients, toxic materials, and organic and suspended solids that are not removed by the secondary stage (Bouki *et al.,* 2013).

Wastewater treatment plants can remove 83-95% of microplastics, but that still leaves a substantial amount of microplastics that can enter the marine environment directly or indirectly (Eckert *et al.,* 2017; Weideman *et al*., 2019; Weideman *et al*., 2020). The detected small

microplastic amounts in WWTP effluent are, therefore, substantial due to the large and continuous amount of WWTP effluent discharged into the aquatic environment annually (Pareao *et al.,* 2020).

Wastewater comprises large quantities of organic and inorganic contaminants, which can potentially interact with the microplastics present, thereby modifying the environmental fate and possibly making microplastics the carriers of these wastewater pollutants, such as hormones, chemicals, pharmaceuticals, and pesticides (Ziajahromi *et al.,* 2016). The major pollutants in wastewater are heavy metals, hydrocarbons, organic matter, detergents, pesticides and herbicides while macronutrients include nitrogen (N), and phosphorus (PO_4) (Akpor, 2011; Akpor *et al.,* 2014).

2.7. Antibiotics

Antibiotics can be described as chemotherapeutic agents that kill or inhibit the growth of microorganisms, such as bacteria, fungi, and protozoa (Kümmer, 2008). Resistance can be described as the capacity of bacteria to adapt to their environment and survive (Kümmer, 2008). The first antibiotics were of natural origin while they are currently obtained through chemical synthesis (Bouki *et al.,* 2013). Antibiotics have become one of the most important medical intervention and prevention methods in the current century. Large amounts of antibiotics are released into the aquatic environment due to the incomplete metabolism in humans and animals, which are released into the municipal wastewater and ultimately into the aquatic environment.

The misuse and overuse of antibiotics in humans and animals contribute to the development of antibiotic resistance (Allen *et al*., 2010; Schwartz *et al.,* 2003). This resistance can arise through clonal dissemination of pathogenic bacteria, which possess resistance mechanisms that alter target molecules due to mutational events (Allen *et al*., 2010). Most known antibiotic-resistance genes are found on transposons, integrons and plasmids, which can be mobilised and transferred to other bacteria (Allen *et al.,* 2010).

The widespread use of antibiotics in medicine, agriculture and animal husbandry is indicative of the pressure exerted on bacteria. The agricultural needs and animal husbandry provide a direct route for antibiotic-resistant bacteria into the environment through manure and muck (Schwartz *et al.,* 2003). According to Bouki *et al.* (2013), once antibiotics enter the ecosystem, the evolution of the community structure can be affected, which in turn influences the ecological function of the aquatic ecosystem.

Antibiotics can be divided into different classes depending on their “strength”, namely Beta-lactams, Macrolides, Glycopeptides, Oxazolidinones, Aminoglycosides, Tetracyclines,

Sulphonamides, Sulphanilamide and Quinolones (Kümmer, 2008; Etebu & Arlkekpar, 2016). Antibiotics within each class will generally show similar patterns of effectiveness, toxicity and allergic reactions (Etebu & Arlkekpar, 2016).

Antibiotic strength refers to the effectiveness or potency of an antibiotic in inhibiting the growth of bacteria and can be influenced by the chemical structure, action mechanism, spectrum, and susceptibility of the bacterium (Cost *et al.,* 2011). Antibiotics can, therefore, inhibit cell wall and protein synthesis, bacterial DNA (Deoxyribonucleic acid) and RNA (Ribonucleic acid) disruption and lastly, interfere with metabolic pathways (Peterson & Kaur, 2018; Etebu & Arlkekpar, 2016). Antibiotic resistance mechanisms include the modification of the antimicrobial route by preventing the reach of antibiotics by either actively extruding the antimicrobial compound, decreasing the penetration, or changing the target sites and resistance due to cell adaptation (Munita & Aries, 2016).

Most antibiotic-producing strains carry genes that encode certain resistance to the antibiotics they produce and can be found in the same gene cluster as the antibiotic biosynthesis pathway genes (Allen *et al.,* 2010). Antibiotic biosynthesis gene clusters encode one of more potential resistance proteins that are either compound-specific or multifunctional (Allen *et al.,* 2010). According to Balcázar *et al.* (2015), antibiotic-resistance genes tend to accumulate in biofilms rather than in the planktonic compartment.

South Africa has a high consumption of different antibiotics; therefore, a large proportion of multidrug-resistant organisms have developed as a result (Faleye *et al*., 2019). It is assumed that antibiotic consumption is reflected in the occurrence and quantities in the inlet water of WWTP, thereby acting as hotspots by means of which to measure and quantify the occurrence of emerging pollutants (Faleye *et al*., 2019).

2.8. DNA transfer mechanisms between microbes in biofilms

Bacteria have developed different mechanisms to render the antibiotics implemented against them ineffective (Schwartz *et al.,* 2003). The genes responsible for the encoding of defence mechanisms are located on the bacterial chromosome or on extrachromosomal plasmids that can be transferred to the next generation through vertical gene transfer (Schwartz *et al.,* 2003). Plasmids can be exchanged amongst different bacteria or different taxonomic affiliations through horizontal gene transfer (HGT) (Schwartz *et al*., 2003; Jiang *et al*., 2022). Horizontal gene transfer and convert silent ARG into functional ARG and, in doing so, spread ARG throughout the microbial pangenome (Jiang *et al*., 2022). Horizontal gene transfer of ARGs is affected by multiple non-abiotic factors, including natural factors (temperature and CO_2), man-made contaminants (pharmaceuticals, antimicrobial agents and nanomaterials) and water

treatment processes (Jiang *et al*., 2022). Through the various HGT mechanisms, such as conjugation, transformation, transduction and vesiduction, ARGs harboured by non-pathogenic bacteria can be transferred to pathogenic bacteria and vice versa (Honceriu, 2022; Balcázar *et al.,* 2015; Soler & Forterre, 2020). Therefore, antibiotics can drive the horizontal transfer of ARGs within the environment. Figure 2.3 illustrates the different HGT mechanisms.

Conjugation is described as genetic material transfer between two bacterial cells within the same environment and can be achieved through two methods such as genetic transfer through a pilus or surface adhesion (Honceriu, 2022). A pilus is used by Gram-negative bacteria, whereas Gram-positive bacteria use surface adhesion during conjugation (Virolle *et al*., 2020; Grohmann *et al*., 2003). According to Hospenthal *et al.* (2022) and Goessweiner-Mohr *et al*., (2013), conjugative pili is used for the connection of the donor and acceptor bacteria that enables the transport of DNA, whereas contact mediated through other types of pili can lead to infection, bacterial motility, microcolony or biofilm formation. Conjugation allows for the transfer of resistance genes between bacteria with large phylogenetic distances, indicating the dissemination of resistance genes from different gene pools (Honceriu, 2022). Conjugation is one of the most efficient pathways to disseminating antibiotic resistance among bacterial cells, where the donor and recipient cells are in close contact with each other; and is mediated by the conjugative plasmids and conjugative transposons (Balcázar *et al.,* 2015). The most important aspect of conjugative plasmids is that they can be exchanged among related and phylogenetically distant bacteria (Jiang *et al*., 2022).

Transformation is the process by which exogenous naked DNA fragments are taken up by pilus-like structures and permanently integrated into the genome of bacteria, either in the chromosome by homologous recombination or incorporated as plasmids but can also serve as a nutrient source, bypassing de novo biosynthesis of nucleotides (Honceriu, 2022; Michaelis & Grohmann, 2023). Transformation can enable bacteria to acquire new genetic traits and adapt to changing environmental conditions, such as promoting antibiotic resistance (Johnston *et al*., 2014; Michaelis & Grohmann, 2023). Transformation can occur between taxonomically diverse bacteria and disseminate antibiotic resistance among phylogenetically distant bacteria (Jiang *et al*., 2022). Unlike transduction and conjugation, transformation is directed by the recipient cell and all the required proteins are encoded in the core genome (Johnston *et al*., 2014). Water treatment processes can eliminate ARB, but the DNA remnants released from the inactive ARB may persist in water environments through the adsorption of cellular debris and, in doing so, provide media for transformation (Jiang *et al*., 2022).

According to Honceriu (2022), transduction occurs when genes from the donor cells are incorporated into the bacteriophage genome and when the genes from the donor cells are

injected into the recipient cell. The ARG-containing DNA from the donor cell is packaged into the bacteriophage capsid, which leads to the bacteriophage genome being released from the donor cell and passed onto a recipient cell (Jiang *et al*., 2022). It has been found that some bacteriophages can transmit DNA fragments larger than 100 Kb, sufficient in size to carry plasmids (Honceriu, 2022). Bacteriophages (shortly designated as phages) are viruses that infect bacteria, they are present in the natural environment and not easily degradable under environmental conditions, which results in a longer lifespan compared to bacteria. Phages possess the ability to acquire ARGs and disseminate antibiotic resistance to the surrounding bacteria (Michaelis & Grohmann, 2023; Jiang *et al*., 2022).

Vesiduction is described as the secretion of membrane vesicles from a doner cell surface that transport ARG-containing DNA into the recipient cell's cytoplasm by fusing with the cytomembrane (Soler & Forterre, 2020; Jiang *et al*., 2022). These membrane vesicles can prevent DNA from degradation by DNases (Deoxyribonuclease) or other environmental factors (Liu *et al*., 2024). Vesiduction plays an essential role in the horizontal transfer of ARGs in bacterial biofilms in aquatic environments (Liu *et al*., 2020).

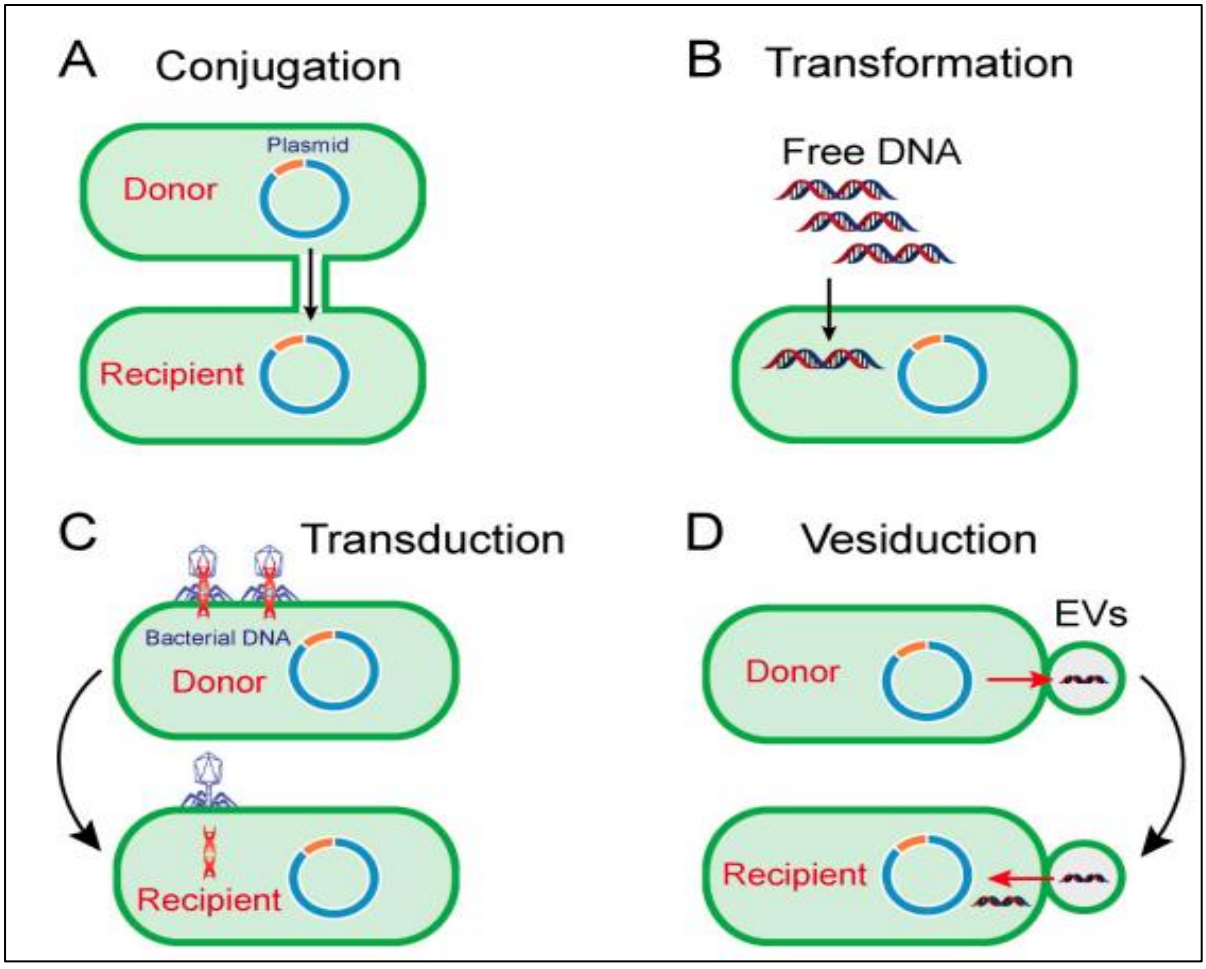


Figure 2.3: Mechanisms of horizontal gene transfer, Conjugation (A), Transformation (B), Transduction (C), and Vesiduction (D) (adapted from Honceriu, 2022; Liu *et al*., 2020).

Horizontal gene transfer is mediated by mobile genetic elements, which play a significant role in the evolution and adaptation of bacterial species to changing environmental conditions (Balcázar *et al.,* 2015). Balcázar *et al.* (2015) and Patridge *et al*. (2018) described mobile genetic elements (MGE) as DNA segments encoding a variety of enzymes and proteins that mediate their movements within the host genome or between bacterial cells. Interchange of DNA fragments between a cell donor and receptor takes place through conjugation, transformation, or transduction, whereas intracellular movement is facilitated by integrons and transposons (Balcázar *et al.,* 2015). The elements that move bacterial genes from one bacterial cell to another, HGT, are conjugative plasmids, they can promote their transfer and the transfer of other plasmids from one bacterial cell to another (Bennett, 2008). Horizontal gene transfer by conjugative plasmids is common within biofilms due to the high density of bacteria and, accordingly, the probability of two suitable bacterial cells near each other (Schwartz *et al*., 2003).

Plasmids serve as the foundation for assembling and rearranging gene arrays (Bennett, 2008). Plasmids are extrachromosomal DNA molecules capable of autonomous replication (Carattoli, 2013). Through the accumulation of beneficial genes, such as ARG, aided by various recombination systems, these platforms enable bacteria strains to adapt to dangerous environments, including developing antibiotic resistance (Bennett, 2008; Carattoli, 2013). Plasmids are replicated independently of the main bacterial chromosome and do not contain the core genes needed for cell growth and multiplication but rather contain genes that are advantageous in certain environmental conditions (Bennett, 2008; Zhang *et al*., 2023). Therefore, plasmids carry a wide variety of genes, including those that confer antibiotic resistance and resistance to numerous toxic heavy metals and enzymes that increase the nutritional ability of the cell (Bennett, 2008). Plasmids can therefore promote the horizontal transfer of resistance determinants among bacteria of different species from different genera and kingdoms depending on the host range, conjugative properties and conjugation efficiency (Carattoli, 2013).

Resistance plasmids could be described as plasmid that carry one or more antibiotic-resistance genes within a microbe (Bennett, 2008; Rowandowicz *et al*., 2018; Carattoli, 2013). Multiple physically linked genetic determinants, conferring resistance to different antibiotic classes can be found on the same plasmid (Carattoli, 2013). The majority of the resistance plasmids are conjugative in nature; they encode functions necessary for cell-to-cell DNA transfer, while others are mobilizable when helped by a conjugative plasmid co-resident in the cell (Bennet, 2008). Plasmids conferring multi-drug resistance are large (>50 Kb), self-conjugative and encode sophisticated mechanisms controlling their copy number and regulating the replication rate (Carattoli, 2013).

Mobilizable plasmids lack the genes that encode functions that enable cells to couple before DNA transfer, but they do encode for the transference of their DNA with a helper plasmid (Bennet, 2008; Zhang *et al*., 2023). Mobilizable resistance plasmids are less than 10 Kb in size and encode for a few genes, including the resistance genes, compared to conjugative plasmids that are bigger, 30 Kb or more, thereby reflecting the amount of DNA needed to encode for the conjugation functions that will allow cell-to-cell coupling, especially in Gram-negative bacteria (Bennett, 2008). Mobilizable plasmids are seen to have limited mobility and the ability to encode for relaxases and *oriT* (Zhang *et al*., 2023). The transfer of mobilizable plasmids with only an *oriT* relies on the assistance of specialized conjugative plasmids to carry the relaxases to aid in the recognition and cleaving of oriT on mobilizable plasmids (Zhang *et al*., 2023). Gram-negative bacterial coupling is mediated by an external filamentous appendage called a sex pilus, which aids in the joining of the donor and recipient cells, which is then retracted into the donor, and a DNA transfer pore forms to bridge the cytoplasmic compartments of the conjoined cells (Bennet, 2008). A common plasmid, Residence Plasmid 1 (RP1 but also known as RP4 and RK4), was first identified in *Pseudomonas aeruginosa* and can transfer to Gram-negative bacteria (Bennett, 2008).

The spread of multi-drug resistance has been achieved through the acquisition of preexisting determinants followed by the amplification in response to the selection (Patridge *et al*., 2018). Mobile genetic elements can be classified into two groups: elements that can move from one bacterial cell to another, such as resistance plasmids and conjugative resistance transposons; and elements that can move from one genetic location to another in the same cell, including resistance transposons, gene cassettes and ISCR (Insertion Sequence Common Region)-promoted gene mobilization (Bennett, 2008; Patridge *et al*., 2018). The ISCR elements can pick up adjacent sequences by rolling circle replication and can be seen as aiding the movement of antibiotic resistance genes (Patridge *et al*., 2018).

Resistance transposons can be seen as jumping gene systems that incorporate a resistance gene within the element, they come in different forms and are distinguished by their structure, genetic relatedness, and transposition mechanism (Bennett, 2008). Transposons can be grouped with a set of mobile elements, transposable elements, that encompass small cryptic elements called insertion sequences (IS) (Bennett, 2008; Patridge *et al*., 2018). Transposons differ from an IS element, due to the encoding of one or more functions that change the cell phenotype in a predicted fashion (Bennett, 2008; Patridge *et al*., 2018). Insertion sequences can be divided into groups depending on their active site motifs and whether the transposon is conservative or replicative (Patridge *et al*., 2018).

Transposons are either modular systems, referred to as composite transposons, constructed from IS elements and a central DNA sequence, which alter the cell phenotype expression or as a complex system, where transposition and non-transposition functions have not been assembled in a modular fashion (Bennett, 2008; Patridge *et al*., 2018). Transposons play an important role in the transmission of accessory structures, such as metabolic pathways or resistance genes (Honceriu, 2022). Tn*5* is a well-known transposon that encodes resistance towards aminoglycosides, including kanamycin (K30) and neomycin, as well as Tn10, which encodes resistance towards tetracycline, and both compound elements can be found in Gram-negative bacteria belonging to the Enterobacteriaceae family (Bennet, 2008).

An IS element-dependant resistance transposon requires two copies of the same IS elements for flanking terminal structures, direct or inverted repeats, to the central section that contains the antibiotic-resistance genes to be conferred (Bennett, 2008; Patridge *et al*., 2018). An inverted arrangement of IS elements is genetically more stable than the direct repeat arrangement, but with less opportunities to migrate to other IS element sites (Bennett, 2008). A two-stage homologous recombination process occurs, whereby a part of the composite structure is excised from the existing site by a single crossover between the IS elements copies, releasing circular double-stranded DNA species compromising the central section of the composite resistance transposon and a copy of the IS element, the other remaining at the original genetic location (Bennett, 2008). The released DNA can then be rescued by reversing the first recombination, through homologous recombination, involving a single crossover and using the IS sequence copy on the free intermediate and another copy at the new location which results in a new composite transposon at the new site (Bennett, 2008; Zhang *et al*., 2023). The positives of these arrangements are that the transposition modules, IS elements, retain the ability to transpose as individual elements and as part of the compound structure (Bennett, 2008).

When a plasmid arrives within a bacterial cell, either by conjugation or transduction, transposons can further mobilize genes for resistance within the new plasmid by copying these genes onto a new plasmid or into the bacterial chromosome, thereby creating a new dynamic in which resistance genes taken from newly acquired plasmids are transferred to other plasmids and ultimately increasing the potential of resistance genes to be transmitted through the conjugation (Honceriu, 2022).

Bennet (2008) described bacterial integrons as gene-capturing systems that utilize site-specific recombination and are found on plasmids or transposons. They comprise a specialized recombination system consisting of a gene, *int1*, which encodes for a site-specific recombination enzyme called integrase, and a site at which short DNA sequences and gene

cassettes are inserted by the integrase (Bennett, 2008; Deng *et al*., 2015; Patridge *et al*., 2018). Much of the resistance integrons are described as class 1 integrons by containing a distinctive structure that comprises two terminal invariable regions termed constant sequences (CS) and a highly variable central section (Bennett, 2008). Integrons are more commonly found in Gram-negative bacteria and can be divided into five classes: 1, 2, 3, 4 and 5 (Honceriu, 2022). Class 1 and 3 integrons are present in the proteobacteria group within the soil and freshwater environments, whilst class 2 integrons are found within the marine gamma proteobacteria group (Deng *et al*., 2015). The Class 1 integrons are a common contributor to the distribution of antimicrobial resistance and the prevalence ranges from 22 to 59%, identified in *Acinetobacter*, *Aeromonas*, *Burkholderia*, *Campylobacter*, *Enterobacter*, *Escherichia, Providencia, Pseudomonas*, *Salmonella, Stenotrophomonas, Vibrio,* and *Klebsiella* genera (Honceriu, 2017; Deng *et al*., 2015). Class 4 integrons were used to refer to an integron found within the *Vibrio cholerae* chromosome and contain a large array of cassettes (Patridge *et al*., 2018).

The high cell density and close contact among other cells within the biofilm matrix, together with increased genetic competence and accumulation of MGE, convert these habitats into an optimal scenario for the acquisition and spread of ARG (Balcázar *et al.,* 2015; Goessweiner-Mohr *et al*., 2013). The various methods for HGT can, therefore, be seen as essential for the spread of ARG within biofilms in aquatic habitats.

2.9. Microplastics and biofilm interaction

Microorganisms attach themselves to surfaces, either biotic or abiotic, forming a complex matrix of biopolymers, known as biofilms, that aid in protection from environmental hazards (Balcázar *et al*., 2015). Biofilms may comprise single bacterial species or a complex and diverse community of micro-organisms embedded in an extracellular matrix of polysaccharides, exudates, and detritus (Balcázar *et al.,* 2015). A matrix containing adhesive proteins, an Extracellular Polymeric Substance (EPS), between the organisms develops, causing a mesh-like structure to form and nutrients to be distributed evenly amongst the organisms as well as genetic material (López *et al.,* 2010). According to Tumwesigye *et al.* (2023), EPS can determine the structural and functional integrity of the biofilms formed and can affect the morphology, density, and chemical properties of the microplastics. These biofilms formed can promote chemical and physical adsorption onto microplastics through trapping, accumulation, and multiple binding sites (Tumwesigye *et al.*, 2023).

In aquatic habitats, biofilms not only develop on benthic substrata but also on floating macro- and micro aggregates (Simon *et al.,* 2002; Tu *et al.,* 2020). Biofilms occur on submerged

surfaces in natural and man-made systems, providing an optimal environment for the growth, activity, and bacterial interaction between different species (Balcázar *et al.,* 2015). Biofilms can also provide a shelter to adjust to external stressors, metabolic interactions, and genetic exchange between bacterial species. Biofilm formation is divided into the early stage (1-14 weeks), which is seen as the irreversible attachment to the surface; the mid-stage (14-35 weeks) or bacterial division and creation of the extracellular matrices; and the late stage (35-45 weeks), the disintegration of the matrices based on changes within the biofilm combined with environmental stressors (Tu *et al.,* 2020; Sahoo *et al.,* 2021).

Biofilms show increased survival and resistance to environmental stressors through the protection of the extracellular polysaccharide matrix. In biofilms, bacterial cells are 10 to 1000 times less susceptible to specific antimicrobial agents compared to their planktonic counterparts (Balcázar *et al.,* 2015). The reduced susceptibility can be caused by different factorial combinations, the antibiotics poorly penetrating the polysaccharide matrix, the presence of cells that show a resistant phenotype and the presence of non-growing cells or either cell that triggered the stress response under unfavourable conditions within the biofilm matrix (Balcázar *et al.,* 2015). These protective mechanisms act synergistically with those responsible for conventional resistance linked to antibiotic-resistance genes in bacterial genomes or extrachromosomal elements, yielding an increased resistance of biofilms towards antimicrobial compounds (Balcázar *et al.,* 2015).

According to Balcázar *et al.* (2015), biofilm formation may also result in a defensive reaction towards antibiotic presence. Biofilm formation on microplastics can occur in two stages, namely, the absorption of organic molecules and the attachment of bacteria (Lobelle & Cunliffe, 2011). With the attachment of the bacteria, there is a film formed which can modify the surface properties and influence the bacterial colonization of these particles (Galgani *et al.,* 2018). Once the surface properties are changed, the adhesion of other micro-organisms is made possible and can lead to a complex biofilm containing different bacteria (Galgani *et al.,* 2018; Nath *et al*., 2023).

According to López *et al.* (2021) and Sahoo *et al.* (2021), a possible reason for the increased resistance to environmental stressors is the increased amount of persister cells within the biofilm. Persister cells are resistant to a variety of antibiotics and are non-dividing; it has been proposed that persister cells are protected through expressing toxin-antitoxin systems, where the target of antibiotics is blocked by toxin modules (López *et al.,* 2021). The presence of an extracellular matrix protects the constituent cells from external aggressors and acts as a diffusion barrier for small molecules, nutrients, vitamins, and minerals (López *et al.,* 2021).

Biofilm formation can be dependent on the surface structure, the chemical composition, environmental conditions such as light availability, salinity, temperature, and pressure, as well as the movement and location of the plastic particles in the aquatic environment (Harrison *et al.,* 2018). Colonization or biofouling can influence the buoyancy of the floating plastic particles, causing neutral or negative buoyancy and resulting in deeper settling within the water column (Schlundt *et al.,* 2019). According to Tumwesigye *et al.* (2023), EPS can determine the structural and functional integrity of the biofilms. Therefore, biofilm populations experience complex diffusional gradients of nutrients and waste products that result in a wide array of physiological states and growth rates (France *et al.,* 2019). Attachment mechanisms have been developed; surface structures have been formed, for example, filli, cilia, pili, and fimbriae, as well as to regulate the EPS to increase the probability of attachment (Rummel *et al.,* 2017). *E. coli* produces type 1 fimbriae that are equipped to adhere to mannose-containing receptors and are commonly found on plastic surfaces (López *et al.,* 2021).

Due to anthropogenic activities and the continuous discharges from WWTP into the aquatic environment, the microorganisms inhabiting these environments are exposed to a low but constant concentration of a wide range of chemical pollutants that can alter their behaviour at various stages (Balcázar *et al.,* 2015). Environmental concentrations of antimicrobial compounds are several orders below the minimum inhibitory concentration but can act as signalling molecules, mediating a wide variety of cell processes, including gene transcription and expression, quorum sensing, inter- and intra-species communication, and biofilm formation instead of causing cell death (Balcázar *et al.,* 2015). The low antibiotic concentrations may also trigger different stress responses that may accelerate HGT and the spread of ARG in a wide variety of bacterial species.

Biofilms specialised cell types arise due to differences in gene expression but not gene composition (López *et al.,* 2021). The extracellular conditions influence cell differentiation within communities, the gradient formation of nutrients, oxygen or electron acceptors throughout the biofilm creates microenvironments to which cells respond by altering their gene expression (López *et al.,* 2021).

Biofilm formation is considered a protective mechanism against external assault; specific extracellular cues can, therefore, regulate the activation of metabolic pathways that can lead to biofilm formation (López *et al.,* 2021). Thus, external cues can come from diverse sources, and the bacterial community itself can produce and secrete signals known as autoinducers (López *et al.,* 2021). Autoinducers accumulate extracellularly with a correlation towards population density; high concentrations of autoinducers trigger signal transduction cascades that lead to multicellular responses in the bacterial population (López *et al.,* 2021). This cell-

to-cell communication is known as quorum sensing and controls a large number of developmental processes, including biofilm formation (López *et al.,* 2021).

Within Gram-negative bacteria, the quorum sensing systems respond to a class of autoinducers and are classified as acyl homoserine lactones (AHL), compared to Gram-positive bacteria where the autoinducers are often peptides and are detected outside the cell (López *et al.,* 2021). For autoinducers to be detected extracellularly, autoinducer molecules are sensed by membrane-associated sensor kinases, which in return activate the cognate response regulators by phosphorylation that then activates the target gene expression (López *et al.,* 2021).

2.10. Microplastics act as vectors

The vector effect of microplastics may modify the behaviour of microplastics in the environment due to the interaction between the absorbed/attached chemical or biological agent (Tumwesigye *et al.,* 2023). Plastic materials have different surface characteristics, chemical composition, carbon chains, functional groups, and crystallinity, which play a role in the interaction between microbes and the environment (Tumwesigye *et al.,* 2023). The plastic material contamination interaction can be by van der Waals, hydrophobic, hydrogen bonding, electrostatic interaction-repulsion forces, halogen bonding, π-π interaction or partition effect (Tumwesigye *et al.,* 2023).

Hydrophobic organic pollutants are absorbed by hydrophobic microplastics (Duis & Coors, 2016). Microplastics present a large surface-to-volume ratio and chemical composition; microplastics can accumulate waterborne contaminates, including metals, persistent, bio-accumulative, and toxic compounds, and transfer to the surrounding biota (Wagner *et al.,* 2014). Microplastics are enriched with contaminates such as hexachlorinated hexanes, polycyclic aromatic hydrocarbons (PAHs), polychlorinated diphenyl ethers (PBDEs) and polychlorinated biphenyls (PCBs) (Duis & Coors, 2016). PP, PE, PS and PET are common vectors of these chemicals.

Microplastics can adsorb organic compounds, such as pharmaceuticals, persistent organic pollutants (POP) and chemical trace elements (Dyachenko *et al.,* 2016; Sheridan *et al*., 2022). The sorption and desorption process depends on the polymer type. For instance, phenanthrene reached higher equilibrium concentrations of PE than PP and PVC particles (Duis & Coors, 2016). Over 160 pharmaceutical compounds belonging to commonly used medication groups, such as antibiotics, anti-inflammatory drugs, and heart disease medicine, have been detected in open surface waters, indicating inadequate removal from the WWTPs (Tumwesigye *et al.,* 2023).

The sorption of pharmaceutical compounds by micro-plastics is regulated by the KOW (octanol-water partition coefficient), polymeric properties of the microplastics and the matrix conditions, for example, the salinity and total dissolved solids present (Tumwesigye *et al.,* 2023). The bioaccumulation capacity of pharmaceuticals also exhibited positive interactions with the chemical properties of these substrates (Ohoro *et al.,* 2021). Chronic exposure to antibiotics promotes and maintains a pool of resistance genes in natural microbial communities within the environment (Balcázar *et al.,* 2015). Therefore, biofilms confer benefits to the members of the community, including recalcitrance, and the ability of pathogenic biofilms to survive in the presence of high concentrations of antibiotics (Uruén *et al.,* 2020). Biofilm recalcitrance consists of two phenomena, namely antibiotic resistance and antibiotic tolerance (Uruén *et al.,* 2020). Li *et al.* (2017) analysed the adsorption of five antibiotics on five microplastics in freshwater systems and found that polyamide particles could be used as a transport for antibiotics in freshwater environments due to their porous structure and hydrogen bonds.

According to Duis and Coors (2016) and Allen *et al*. (2010), the concentrations of pollutants in microplastics are several magnitudes higher than in the surrounding water, as well as the ingestion by a variety of organisms; microplastics may lead to increased uptake of pollutants by aquatic organisms. Gouin *et al.* (2011) and Koelmans *et al.* (2013) studied the bioaccumulation of contaminants into the environment; they concluded that the microplastic contribution to bioaccumulation is minimal due to the high affinity of the microplastics to the pollutants, which prevents the transfer of contaminants to the environment.

2.11. Antibiotic-resistance genes and the plastisphere

The abundance of microplastics and antibiotics in aquatic environments has led to the rise in ARG and ARB around the globe. The plastic-attached microbes, referred to as the plastisphere, are therefore exposed to the surrounding pollutants and thereby affect the biofilm community surrounding the microplastics (Gouin *et al*., 2011; Koelmans *et al*., 2013; Stevenson *et al*., 2024). The plastisphere has been found to be distinct from their surrounding environment and other natural debris but also acts as a vector for ARB and potential pathogens (Stevenson *et al*., 2024; Zettler *et al*., 2013). The attachment of phenotypes may increase the infectivity of microorganisms due to various metabolic pathways and diversity within the microbial community (Stevenson *et al*., 2023; Stevenson *et al*., 2024).

Plastisphere communities have been found to differ from free-living communities within the same environment (Stevenson *et al*., 2024; Zettler *et al*., 2013). McCormick *et al*. (2016) studied the bacterial assemblages on microplastics in streams and compared the bacterial

assemblages found on natural debris within the rivers. The study revealed that the community richness and diversity were lower on microplastics than on natural debris, but sequences representing *Arcobacter* were more abundant on the microplastic than on the natural debris; this genus contains pathogenic species and is indicative of sewage water (McCormick *et al*., 2016).

According to Santos-Lopez *et al*. (2021), ARB within a biofilm is ultimately a result of natural selection and evolution; the proximity of microbial cells in microplastic biofilms and the transfer mechanisms between microorganisms, even those without ARG can lead to the spread of ARG within the plastisphere. More studies are needed to determine the impact of ARG and ARB in biofilms surrounding microplastics in freshwater systems.

2.12. Detection and characterisation of microplastics and biofilms in freshwater systems

Detecting and identifying microplastic particles in the aquatic environment is complex and ununified (Azevedo-Santos *et al*., 2021; Bassiouni *et al*., 2015). The absence of a standard method for sampling and quantifying microplastics has resulted in several studies publishing several methodologies for identifying microplastics within different environments, such as sediment (Möller *et al*., 2020; Koyuncyoğlu *et al*., 2021), freshwater environments (Qiang *et al*., 2021; Talbot & Chang, 2022), marine environment (Wang *et al*., 2015), and WWTPs (Ziajahromi, 2018; Dyachenko *et al*., 2016). While the discovery of microbes surrounding these plastic debris using scanning electron microscopy provided a momentary view of the “plastisphere”, the isolation methods for the detection and identification of microplastics and the surrounding microbes with the use of scanning electron microscopy presented futile (Schlundt *et al*., 2020). Therefore, various phenotypical and molecular techniques are coupled with *in-situ* hybridization for the visualisation and molecular identification of the plastisphere (Stevenson *et al*., 2023).

2.13. Visualisation techniques used for the plastisphere

The plastisphere can be visualised by various methods, including scanning electron microscopy (SEM), fluorescence *in-situ* hybridization (FISH) and confocal laser scanning microscopy (CLSM). (Golding *et al*., 2016; Day *et al*., 2022). Visualisation of the plastisphere provides better insight into microbial colonisation of particles, transfer mechanisms and a better understanding of the microbial world present in freshwater systems.

2.13.1. Scanning electron microscopy

Scanning electron microscopy is regarded as an effective method in the analysis of organic and inorganic materials on a nanometre to micrometre scale. Scanning electron microscopy magnification reaches 300000 x and provides a more detailed field with grey-scale images (Mohammed and Abdullah, 2018). Scanning electron microscopy is based on a focused beam of electrons that scan the sample, resulting in atom interactions which leads to a three-dimensional surface topography and morphology of the microbial cells and surface of the substrate (Pallares-Rusiñol *et al.,* 2023). Scanning electron microscopy can be carried out with as little as 5000 total particles per sample. Scanning electron microscopy sample preparation is subject to fixation, drying, staining and sputter coating processes before a high-resolution image can be taken (Golding *et al.,* 2016). Scanning electron microscopy disadvantages include a conducting surface being required for adequate contrast and reduction of charging of the small organic particles, where, at magnifications higher than 1000, particle dehydration is a time-consuming and intensive process, being costly and can influence image quality (Golding *et al.,* 2016). Scanning electron microscopy is advantageous in providing a clear three-dimensional picture of the plastic surface and the microbial assemblages on the substrate surface.

2.13.2. Fluorescence *in-Situ* hybridization

Fluorescence *in-situ* hybridization can also be used to visualize the plastisphere. A combination of SEM and FISH can result in the spatial and taxonomic resolution of the microbial community on the substrate, however, the heterogenous communities surrounding the substrates may present a challenge when applied to the biofilms surrounding the microplastics (Schlundt *et al.,* 2019). Schlundt *et al.* (2019) made use of an epifluorescence microscopy technique; combinatorial labelling and spectral imaging–fluorescence *in-situ* hybridization (CLASI_FISH), which uses fluorophore-labelled oligonucleotides to identify the microbial taxa along with 16 fluorophores, whose spectral signatures are then distinguished through fluorescence spectral imagining. This technique allowed for the distinction between several microbial groups within one image, showcasing the inter and intradomain interactions in the plastisphere (Schlundt *et al.,* 2019).

2.13.3. Confocal laser scanning microscopy

Confocal laser scanning microscopy is used to decipher biofilm spatial structure and associated functions by use of eliminating unfocused fluorescent signals by collecting the focal plane with a resolution compatible with single-cell visualization (Azeredo *et al.,* 2017). Multi-

acquisitions of such planes at different depths in the sample, combined with dedicated image analysis, represents the 3D architecture of the sample and obtaining quantitative structural parameters, such as the biofilm bio-volume, thickness, and roughness (Azeredo *et al.,* 2017). Confocal laser scanning microscopy makes use of fluorescent probes with unique specifications and nucleic acid dyes, SYTO-9 or SYBR-Green, to label the microbial cells within a biofilm; FISH can also be used concurrently for the identification of specific microorganisms (Azeredo *et al.,* 2017). Cellular death within a biofilm can be investigated through the life/death mixture of CLMS, SYTO-9 (cell-permeant) and red propidium iodine (cell impermeant); the compromised bacteria will appear yellow or red, while the viable cells will appear green (Azeredo *et al.,* 2017). Doroshenko *et al.* (2014) made use of CLSM to investigate the effect of antimicrobial agents on the call viability in biofilms.

2.14. Phenotypical techniques used for the characterization of the plastisphere

Phenotypical techniques can be used to characterize the various bacteria within the plastisphere. Additionally, they facilitate the direct measurement of key phenotypes, such as antimicrobial resistance and pathogenicity, which molecular-based approaches can only infer (Stevenson *et al*., 2023). Pathogenicity is the potential ability of organisms to cause disease within a host organism and can be determined by a combination of factors, referred to as virulence factors (Sharma *et al.,* 2017; Wu *et al*., 2008).

Virulence factors can be defined as the molecules that assist the bacterium to colonize the host at a cellular level and overcome host defences, including chemicals that can cause tissue degradation, adherence factors, attachment structures of bacterial cells such as pili, fimbria, capsules, specialized adhesion molecules, and lastly invasion factors (Leitão, 2020). Invasion factors are surface components that allow the bacterium to invade the host cell and can be encoded on plasmids or chromosomes (Ahmad *et al.,* 2010; Peterson, 1996). Capsules are seen as protective barriers against phagocytosis and opsonization, chemicals that can cause tissue degradation. (Ahmad *et al.,* 2010). Virulence factors are either chromosomally encoded or obtained through genetic mobile elements (Leitão, 2020).

Virulence is therefore defined as the ability of an organism to infect the host and cause disease (Sharma *et al.,* 2017; Wu *et al*., 2008). Thus, virulence is measured to a relative standard, such as another microbe or host (Casadevall, 2017). Virulence factors enable a host to replicate and disseminate within a host, in part subverting or eluding host defences (Cross, 2008). Virulence is measured by the lethal dose that kills 50% of the host within a specific time

frame but also through cytopathology and examining the virulence factors (Cross, 2008; Willey *et al.,* 2011).

Bacteria depend on a plethora of virulence factors in determining the pathogenic potential of microorganisms, where the various virulence factors are influenced by multiple genes. Therefore, virulence testing can be seen as a multitude of differential techniques that aid in determining the pathogenic potential of a microbial isolate. Extracellular enzyme abundance assists with determining the potential virulence of a bacterium and can include, proteinase, lipase, deoxyribonuclease, hemolysin, gelatinase and phospholipase for example (Leitäo, 2020). In this study, the extracellular enzymes used include, lipase, proteinase, hemolysin and deoxyribonuclease.

2.14.1. Gram staining

Gram staining aids in the differentiation and initial classification of unknown bacterial isolates through cell wall structure comparison (Beveridge, 2009). Gram staining enables the separation of bacteria into two groups, Gram-positive and Gram-negative bacteria. Gram-positive bacteria retain the violet stain, whereas Gram-negative bacteria retain the safranin stain.

Gram-positive and Gram-negative bacteria differ from each other, Gram-negative bacteria contain a thicker peptidoglycan layer, which prevents permeability of antibiotics in which fewer multibarrier transportation occurs compared to the peptidoglycan layer in Gram-positive bacteria, seeing as a thinner peptidoglycan layer is present, therefore allowing more easily cross-membrane transportation (Blair *et al.,* 2015). Some bacteria, after staining with the Gram stain, can present a pattern called Gram-variable, where a mix of pink and purple cells are seen (Smith & Hussey, 2005). A problem may arise in the phenotypic characterisation of the genera *Actinomyces, Arthrobacter, Corynebacterium, Mycobacterium* and *Propionibacterium* due to the cell wall breakage during cell division, resulting in Gram-negative staining of these Gram-positive cells (Smith & Hussey, 2005).

2.14.2. Haemolysis assay

Haemolysin is responsible release of haemoglobin and other intracellular components from erythrocytes to the surrounding plasma after cell membrane disruption (Lippi *et al.,* 2008; Willey *et al.,* 2008). There are different types of haemolysins: Alpha (α) haemolysis is described as the reduction of the red blood cell, haemoglobin, to methaemoglobin in the surrounding medium without undergoing complete lysis of the cell membrane resulting in a green or brown discolouration of the blood cells surrounding the colony and is known as partial

haemolysis (Buxton, 2005). Beta (β) haemolysis is defined as the complete lysis of red blood cells, indicated by a clear or transparent zone in the medium surrounding the microbial colony, whereas Gamma (ɣ) haemolysis is seen as a lack of haemolysis, ineffective cell membrane lysis and improper discolouration of the substrate, while a brownish growth on the medium can be seen (Buxton, 2005). Pascoal *et al.* (2018) found that *Candida albicans* haemolysins promote survival and persistence through iron extraction from degraded haemoglobin within the host.

2.14.3. Deoxyribonuclease testing

Deoxyribonuclease (DNase) agar is used as a differential medium that aids in testing the ability of a micro-organism to produce deoxyribonuclease, an exoenzyme that hydrolyses DNA into smaller oligonucleotides (Gerceker *et al.,* 2009). Nucleic acid degradation and fragmentation are promoted by DNase (Pavlov *et al.,* 2004). Buchanan *et al.* (2005) hypothesised that DNase contributes to the overall resistance of a pathogen to phagocytes combined with other virulence factors.

Tetz *et al.* (2009) studied the extracellular DNA of *P. aeruginosa* and *Streptococcus pneumoniae* influencing the biofilm properties by increasing the resistance towards phagocytosis. Deoxyribonuclease is also shown to promote neutrophil-mediated resistance by preventing Streptococci from being killed by neutrophil extracellular traps (NETs) by degrading the neutrophil-mediated antimicrobial system (Florindo *et al.,* 2018).

2.14.4. Proteinase testing

Proteinase is an enzyme that hydrolysis the peptide bonds of proteins within a cell (Zhang *et al.,* 2021). Proteinase (or Proteases) plays an essential role in bioremediation processes, while bacterial proteases are the most remarkable compared to animal and fungal proteases (Das & Prasad, 2010). Proteases regulate the fate, localization, protein activity, modulating protein-protein interactions, and thereby influencing DNA replication and transcription, cell proliferation and differentiation, tissue morphogenesis and remodelling, heat shock, protein response unfolding, and immunity (Lópes-Otín & Bond, 2008). Protease is instrumental in degrading virulence regulators, providing increased tolerance to adverse conditions, host matrix degradation and signalling interference (Frees *et al.,* 2013). *Bacillus* spp. is known as a specific producer of extracellular proteases (Dat & Prasad, 2010).

2.14.5. Lipase testing

Lipases is a lipolytic enzyme that catalyses the hydrolysis of triacylglycerols to diacylglycerols, monoacylglycerols, fatty acids and glycerol (Carrazco-Palafox *et al.,* 2018; Thomson *et al.,* 1999). Bacterial lipase is used for an extensive range of industrial applications. Lipase is responsible for esterification, transesterification, interesterification, acidolysis, alcoholysis and aminolysis conversion (Chandra *et al.,* 2020). The production of lipases can, therefore, affect virulence and microbial health.

2.14.6. Antibiotic susceptibility testing.

The plastic polymers within aquatic environments serve as suitable substrates for the adhesion of microorganisms and the adsorption of antibiotics. The antibiotic resistance within the biofilm needs to be determined to aid in characterising the plastisphere. The performance of antibiotic susceptibility testing (AST) is paramount in determining the susceptibility of antimicrobial agents and the possible drug resistance of microbes (Benkova *et al.,* 2020). Antibiotic susceptibility testing takes 24 hours to obtain bacterial colony growth and a supplementary 24 hours to obtain bacterial characterization through biochemical identification and phenotypic AST (Benkova *et al.,* 2020). Phenotypic AST has numerous advantages compared to genetic testing, namely, the opportunity to predict antibiotic resistance and susceptibility, the ability to enumerate the susceptibility level of a pathogen to the antimicrobial agent, and the possibility of numerous isolate screenings, while disadvantages include prolonged exposure and increased contamination probability (Benkova *et al.,* 2020). Agar disc diffusion is one of the oldest methods for AST but remains one of the most popular techniques. Caruso *et al.* (2024) made use of the Kirby Bauer disc diffusion method to compile the antibiotic susceptibility profiles of the microbial colonisation of PE substrates in marine environments due to the simplicity, low cost and easy interpretation of results.

2.15. Molecular techniques used for the characterization of the plastisphere

A vast majority of microbial species remain uncultivated, and the standard techniques used for cultivation may hinder the microbes' growth (Crossley *et al*., 2020). Therefore, the bulk of micro-organisms are left unexplored (Konopka, 2009). Molecular methods are consequently valuable for environmental studies through determining the diversity and abundance of microorganisms within a particular environment, the relationship between the microbes and the environment and how the influence of these interactions relates to the ecosystem (Oberbeckmann *et al.,* 2014).

Environmental DNA (eDNA) describes the genetic material present in environmental samples, such as air, soil, and water, including whole cells, extracellular DNA, and potentially whole organisms (Ruppert *et al.,* 2019). According to Valentini *et al.* (2015), eDNA investigation offers the advantages of being non-invasive, risk reduction unintentional secondary dispersal of alien species along with increased detection capabilities and cost-effectiveness. Environmental DNA can be used in two scenarios, namely, detecting a single species within the environment, known as eDNA barcoding, or identification of several taxa simultaneously without prior knowledge of potential species in the environment, also known as eDNA metabarcoding (Valentini *et al.,* 2015). Efficient DNA extraction is crucial for the downstream identification of microbial assemblages within an environment. Metagenomic analysis of microbial communities in freshwater environments reveals the genetic capabilities and interplay of waterborne organisms, thereby shedding light on the mechanisms for the production and biodegradation of toxins and other contaminants (Tan *et al*., 2015).

Metabarcoding, through the use of polymerase chain reaction and high throughput sequencing to characterize organisms present in a sample, has been used in a range of ecological implications, such as taxonomic assignments and eDNA analysis by use of a standardized DNA region (Lamb *et al.,* 2018). In eDNA studies, such as this current one, metabarcoding facilitates rapid biodiversity monitoring without the prior isolation of microorganisms (Lamb *et al.,* 2018; Esser *et al*., 2024). Metabarcoding can also assist with discovering new bioindicators, linking microbial community composition and ecosystem function by assessing the functional diversity and activity of these microbial communities (Esser *et al*., 2024). Metabarcoding, therefore, provides insight into community biodiversity and function.

2.15.1. Polymerase chain reaction

Polymerase chain reaction-based methods are commonly used for ARB detection in various environments, such as soil (Zhu *et al*., 2021), freshwater (Witsø *et al*., 2023), marine (Monràs-Riera *et al*., 2024) and sewage (Maday *et al*., 2024). These studies illustrated the high capability of PCR and gel electrophoresis; these molecular methods are more readily used due to the rapid, reliable, and accurate detection of ARB (Witsø *et al*., 2023; Rahman *et al.,* 2013).

Polymerase chain reaction (PCR) is used to amplify short, well-defined DNA fragments from a complex DNA pool (Rahman *et al.,* 2013). Polymerase chain reaction is the most common nucleic acid amplification technique, used for the identification of unknown microorganisms, such as in microcosm studies (Rahman *et al.,* 2013). Other applications of PCR include the direct detection of point mutations, amplification of unknown DNA sequences, related to known

DNA sequences, detection of duplicates and deletions in large genes, and the creation of complementary DNA from RNA (Singh *et al*., 2014).

Limitations may arise, such as primer design being limited by genome knowledge and publicly available sequence databases (Mackay, 2004). The advantages of PCR include sensitivity, versatility, and time efficiency (Ehtisham *et al*., 2016). A template DNA, primers, nucleotides, and DNA polymerase are required to perform PCR (Garibyan & Avashia. 2013). Visualisation of PCR products can be done through the staining of the amplified DNA product by chemical dye, such as ethidium bromide or by labelling the PCR primers with fluorescent dyes prior to PCR amplification (Garibyan and Avashia, 2014).

2.15.2. Sanger sequencing

DNA sequencing is used to obtain the order of nucleotide occurrence in genetic material and, in doing so, provide vital information on bacterial classification. Sanger sequencing is considered the 'gold standard' for DNA sequencing, but due to the limited throughput, speed, scalability, and resolution, next-generation sequencing was developed (Alex *et al.,* 2020; Crossley *et al*., 2020). The 16S rRNA (ribosomal ribonucleic acid) gene is widely used for studying the phylogeny and taxonomy of environmental bacteria due to its presence within all domains of life and frequently existing as a multigene family or operons, the gene sequences encoding for rRNA synthesis are highly conserved and therefore do not change, thereby indicating the evolutionary path of an organism as well as for informatics purposes because of its size (1500 bp) (Janda & Abbott, 2007; Ali *et al*., 2014; Abellan-Scheyder *et al*., 2021). According to Abellan-Scheyder *et al*. (2021) and Yang *et al*. (2016), the 16S rRNA gene is structured in highly conserved regions interspersed with nine variable regions, the conserved regions are used for primer binding allowing for the different bacterial taxa capturing compared to the variable regions that allow for the differentiation of these microbial taxa within an environment.

Sanger sequencing involves PCR amplification, product qualitative detection and separation by gel electrophoresis, purification of the amplicon through ethanol precipitation, sequencing by an amplification reaction and final capillary electrophoresis (Chen *et al.,* 2014). Sanger sequencing limitations include the low-quality sequences within the first 15 – 45 bp due to primer binding and an inability to distinguish single base pair differences in longer segments (Crossley *et al*., 2020). Sanger sequencing of environmentally isolated organisms is limited to the known isolated organisms due to the ineffectiveness of culturing techniques (Chen *et al.,* 2014; Garibyan and Avashia, 2014). According to Wintzingerode *et al*. (1997), cultured microbes only represent a small fraction of the natural microbial communities. Hence, the

species richness and species diversity are underestimated, and thereby, the microbial diversity is unaccounted for. However, Sanger sequencing is the most common method used for the classification and identification of culturable bacteria (Alex *et al.,* 2020). Thus, Sanger sequencing can be beneficial for the identification of culturable bacteria isolated from the plastisphere.

2.15.3. Next-generation sequencing

Studies using next-generation sequencing are providing new insights into the ecology of microbial-mediated processes that influence the surrounding environment (Tan *et al.,* 2015; Boers *et al*., 2019). Next-generation sequencing (NGS) is used to sequence a multitude of genomes simultaneously within a sample to determine the microbial composition of a specific environment (Grada & Weinbrecht, 2013; Boers *et al*., 2019). Next-generation sequencing provides high quality, robustness, high throughput, as well as low interference but requires a long run time (Buermans & den Dunnen, 2014; Hu *et al.,* 2021). Next-generation sequencing can aid in cataloguing the community composition found within water sources for monitoring environmental perturbations and biodegradation along with determining the occurrence and distribution of microbial taxa (Tan *et al*., 2015). Extracted DNA is subjected to PCR amplification using a PCR primer set that targets the taxonomically informative gene, thereafter the resultant amplicons are sequences and characterized by use of bioinformatic tools to determine which microbes are present in the sample and the abundance (Boers *et al*., 2019).

Marques *et al*. (2023) wanted to determine the difference in bacterial communities found on microplastics from estuaries and sea sand. Next-generation sequencing was used to aid in the comparison of these environments. Marques *et al*. (2023) found that the genus *Pseudoalteromonas* had a higher abundance of microplastics from these environments than the environment itself. *Pseudoalteromonas* can harbour potential pathogens and are a common member of the PP and polyethylene biofilms (Marques *et al*., 2023). Using NGS, Frère *et al*. (2018) detected members of the *Vibrio* genus on 80 % of the microplastic samples compared to what was found within the seawater. The study also revealed that microplastics had a higher species richness and diversity compared to seawater (Frère *et al*., 2018). Thus, NGS highlights the “story” of plastic contamination and microbial community within the aquatic environment.

Chapter 3 : Material & Methods:

The processes required for the characterisation of the bacterial biofilm surrounding the microplastics from a simulated freshwater environment include sample collection, microcosm setup, bacterial isolation, SEM, Virulence testing, DNA extraction and data analysis (Figure 3.1).

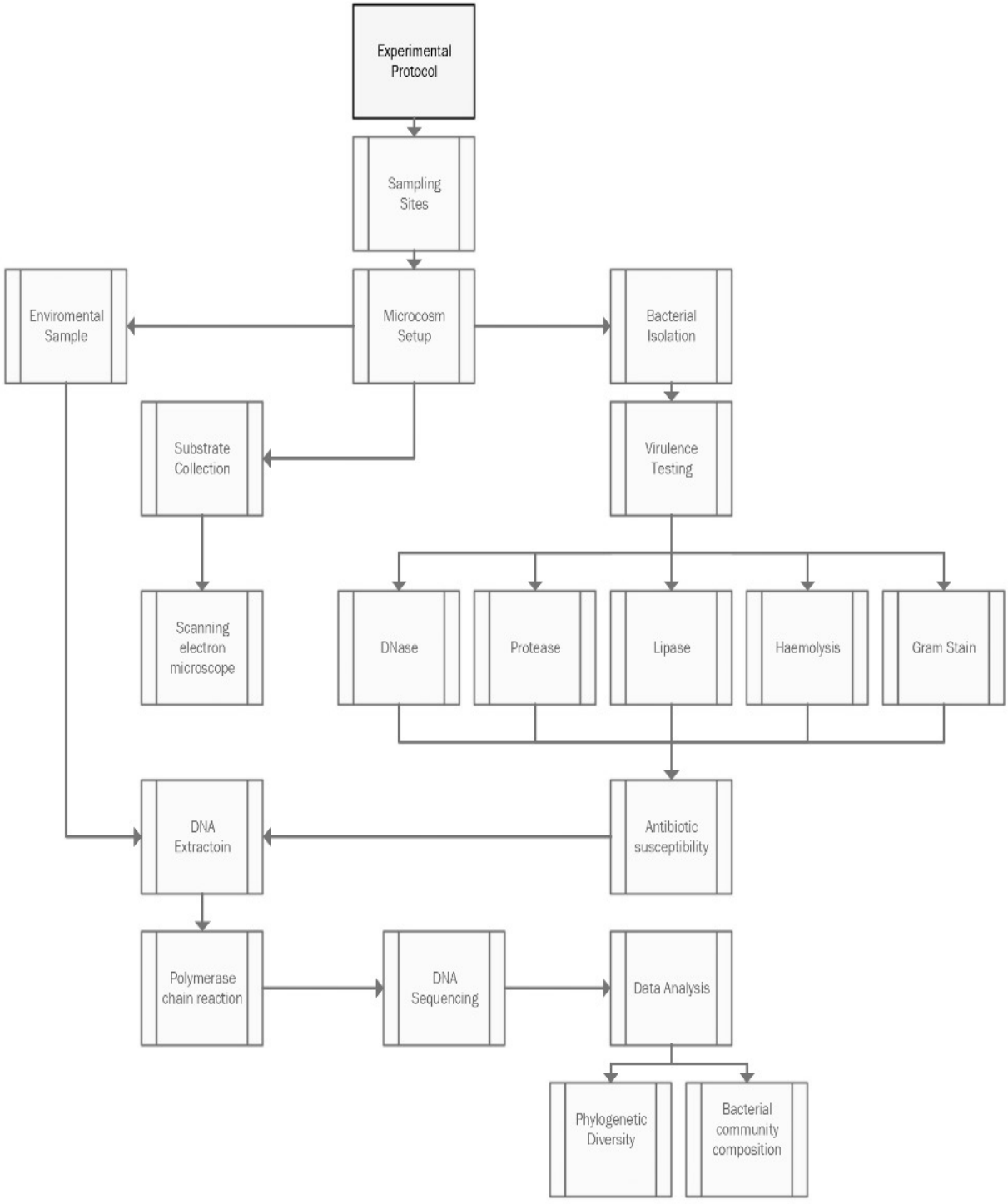


Figure 3.1: Experimental design

3.1 Microcosm setup

Microcosms of the river and WWTP effluent were prepared in 2022 to gain insight into biofilm development and bacterial composition of different microplastics within simulated freshwater environments. Water samples were aseptically collected in sterile 1-litre (1L) bottles from the Mooi River upstream at one sampling point (-26.6833717, 27.0984938) and downstream of the Wastewater treatment facility (Wastewater treatment works, -26.75322358909773, 27.088057006404096) located outside Potchefstroom, North West Province (NWP), SA. Water collection was done by grab sampling, the sterile 1L bottles in the Mooi River stream and at the Wastewater treatment facility were placed at the various sampling points, the bottles were rinsed before being submerged and filled with the river water and wastewater samples. The water samples were collected early in the morning and immediately placed on ice in a cooler for transport back to the lab. The wastewater temperature at the time of the sampling was 23 degrees Celsius (°C).

The different types of microplastics; Nylon, HDPS (high density polystyrene), HDPE, LDPE were selected based on their abundance in the freshwater river systems according to the literature (Lambert & Wagner, 2018; Karkanorachaki *et al*., 2022; Qiang *et al*., 2021; Ramaremisa *et al*., 2022). Different microcosms were constructed for the different types of substrates. The virgin nylon plastic particles (catalogue number 181110) were purchased from Sigma-Aldrich (St. Louis, MO). The remaining virgin plastic particles were purchased from Kruger Commodities, a supplier of plastic compounds and resins in SA.

Toothpicks served as the wood particles, the length of the toothpick was divided into 5 mm increments and cut into these 5 mm increments with sterile scissors. The pre-cut toothpicks (5 cm in length) and post-cut toothpicks (5 mm in length) underwent sterilisation in the autoclave for 30 minutes at 121 °C to eliminate the risk of contamination.

All materials (microplastics and wood) were smaller than 5 mm and underwent sterilisation with 70% ethanol. Sixty pieces of each of the four types of plastics and wood substrate were incubated in a mixture of 200 ml WWTP effluent and 300 ml sterilised river water in a 500 ml Erlenmeyer flask at 23°C for 30 days (Table 3.1). The plastic particles were individually counted before sterilisation. Sterilisation was performed by autoclaving the particles for 30 minutes at 121°C. The microplastics were sorted according to type and amount into numerous sterile petri dishes post sterilization, 70% acetone ethanol was poured into the petri dishes for final sterilization before being placed into the individual microcosms. Each substrate was incubated separately to limit the amount of clustering and aid in direct comparison of biofilm formation and characterisation.

The positive control microcosm consisted of 200 ml WWTP effluent and a mixture of the plastic pieces to simulate the presence of the various plastics within the environment, while the negative control microcosm consisted of 300 ml sterilised river water and the various sterilised microplastics (Qiang *et al*., 2021). The sterilised river water was cooled to 23°C before being placed in the flasks.

The non-plastic control consisted of 60 wood fragments, 300 ml sterilised river water and 200 ml WWTP effluent (Figure 3.2). The non-plastic control was constructed to document the difference in biofilm formation surrounding the inorganic and organic particles. The various microcosms were then capped with sterile foam plugs and foil to prevent contamination (Mahaney & Franklin, 2022). The total time that passed between field sampling and microcosm construction was less than 4 hours to limit the amount of bacterial degradation that could have taken place (Madrid & Zayas, 2007).

Table 3.1: Microcosm composition.

Positive control	• 200 ml WWTP effluent • HDPS, Nylon, HDPE and LDPE particles
Negative control	• 300 ml sterilised river water • HDPS, Nylon, HDPE and LDPE particles
Non-plastic control	• 60 wood particles • 200 ml WWTP effluent • 300 ml sterilised river water
HDPS	• 200 ml WWTP effluent • 300 ml sterilised river water • 60 HDPS particles
HDPE	• 200 ml WWTP effluent • 300 ml sterilised river water • 60 HDPE particles
LDPE	• 200 ml WWTP effluent • 300 ml sterilised river water • 60 LDPE particles
Nylon	• 200 ml WWTP effluent • 300 ml sterilised river water • 60 Nylon particles

The microcosms underwent continuous movement by being placed on a shaking incubator and near a window to stimulate the water flow and movement under environmental conditions (Mahaney & Franklin, 2022). The temperature was kept constant at 23°C.

Figure 3.2: Example of the microcosm vessels (pre-incubation).

Three pieces of each substrate were sampled aseptically on days zero and twenty-eight to observe the microbial colonisation using SEM. The remaining substrates were washed three times with autoclaved distilled water to remove free-floating unattached cells. The samples were stored at -20°C for further analysis. All equipment and utensils used were continuously sterilised throughout the sampling period.

The aseptic collection of the substrates was done by using a sterilized tweezer, a flask full of 70% ethanol, a Bunsen burner and a laminar flow (Bioflow I) cabinet. Each microcosm was placed in the laminar flow (Bioflow I) cabinet on day 0 and day 28 for the SEM substrate collection.

3.2 Sampling sites

The Mooi River flows past Potchefstroom City, which is impacted by agricultural and industrial activities and recreational sites (parks, bird sanctuaries, and shopping malls). The river also flows past a WWTP. This WWTP releases the effluent back into the river, where the river ultimately combines with the Loopspruit and enters the Vaal River.

The freshwater samples were collected in Potchefstroom, NWP, SA. The upstream freshwater sampling site was selected based on no prior influence of the WWTP effluent on the river

along with the surrounding recreational and urban areas. Wastewater samples were collected from the WWTP in Potchefstroom, NWP, SA. The facility relies on filtration for primary treatment and activated sludge for secondary treatment. The effluent was collected after the secondary treatment stage before it was recycled back into the Mooi River.

3.3 Collection of substrates

Ten microplastics of each type were collected aseptically on the different sampling days (Day 0, 3, 7, 14 and 28) and placed onto Reasoner's 2A (R2A) Agar for incubation at 24 hours at 37°C for the characterisation of the bacteria in the biofilm surrounding the microplastics (Bugno *et al*., 2010). Thereafter, one of each different microplastics were selected pre-incubation and on the 28th incubation day and was subjected to fixation for biofilm visualization on the surface using SEM to illustrate the biofilm growth and formation under semi-natural environmental conditions (Relucenti *et al*., 2021). Lastly, the remaining microplastics were placed in 2 ml tubes for DNA extraction and stored at -20°C for further analysis.

The aseptic collection of the substrates was done by using a sterilized tweezer, a full flask of 70% ethanol, a Bunsen burner and a laminar flow (Bioflow I) cabinet. Each microcosm was placed in the laminar flow (Bioflow I) cabinet on various days. The microplastic particles were collected from the microcosm with sterile tweezers, between each particle collection, the tweezers were placed in the 70% ethanol flask and held over the Bunsen burner to sterilize. Thereafter the collected substrates were placed on the R2A agar and streaked over the surface of the plate to disrupt the biofilm community and ensure higher bacterial diversity.

3.4 Scanning electron microscopy preparation

Before the surface observations of the particulates, the microplastics and wood particles were fixed using 3% glutaraldehyde (BDH chem. LTD. England) in cacodylate buffer (Sigma-Aldrich), with a pH value of 6.5 for 10 minutes (Qiang *et al*., 2021). An ethanol dehydration series of 50, 60, 70, 80, 90, and 2x 100% (v/v) was performed on the samples and was followed by a chemical dehydration series of 100% ethanol + hexamethyldisilane (HDMS, Ted Pella, USA) for 5 minutes and left to dry overnight (Pinto *et al.,* 2019). The samples were then sputter-coated with a palladium-gold thin film. The surfaces of the samples were viewed with an SEM/Phenom ProX desktop system (Thermo Scientific, USA) in high-vacuum mode at 15 kV (Mohammed & Abdullah, 2018; Zhou *et al*., 2006) assisted by Willie Landman

3.5 Characterisation of bacteria through virulence testing

Microplastics from the various microcosms were collected using aseptic techniques, as stated above, inoculated onto R2A Agar and incubated for 24 hours on different days (days 0, 3,7, 14 and 28) to provide the best possible chance for bacterial growth (Mahanay & Franklin, 2022; He *et al*., 2023). Various colonies were selected from the R2A agar and inoculated onto sterile nutrient agar plates to ensure the isolation of pure colonies that were used for further identification. The colonies that were selected were chosen based on their shape, colour, size and growth pattern.

Pure colonies were then streak-plated onto nutrient agar as well as several differential media, including DNase agar, blood agar, skimmed milk agar and tryptone soy agar for possible identification. These results provided information about the potential virulence of the organisms. The media preparation was done according to the manufacturer's instructions.

The pure colonies were aseptically transferred and streak-plated on each of the differential media by the use of sterile toothpicks to act as an inoculation loop. The toothpicks were autoclaved at 121˚C for 30 minutes. After each streak was performed, the toothpick was discarded, and a new sterile toothpick was used to complete the streak plating.

3.5.1 Gram staining

Single pure colonies were used for Gram staining. Gram-positive and Gram-negative bacteria were determined based on the microbial cell colour under a microscope. Crystal violet dye was placed onto a fixed microscope slide for 60 seconds, after which an iodine solution was placed onto the fixed slide for 60 seconds before being washed off gently by acetone ethanol (50:50 v: v) (Smith & Hussey, 2005; Bhattacharyya *et al*., 2015). Thereafter, safranin dye was used for 60 seconds as the counterstain. The fixed microscope slide was then viewed under the oil-immersion lens (1000x magnification) (Tripathi & Sapra, 2020). The Gram reactions were recorded and compared to the identification by 16S rRNA gene sequencing.

3.5.2 Deoxyribonuclease agar

To determine the presence of DNase, DNase agar (Oxoid, UK) was prepared according to the manufacturer’s instructions. Plates were spot inoculated with the pure colonies and incubated at 37˚C for 18 - 24 hours. After incubation plates were flooded with 1 M HCl (hydrochloric acid), the formation of a clear zone around the colony was a positive indicator of DNase activity (Pavlov *et al*., 2004; Gundogan *et al*., 2006; Gerceker *et al*., 2009).

3.5.3 Protease

Skimmed milk agar plates were prepared to screen for proteolytic enzyme production. Plates contained 3% (w/v) skimmed milk (Oxoid, England) and Mueller-Hinton agar at 35 g/L (Merck, Germany) (Patidar *et al*., 2013; Sharma *et al*., 2015). Ingredients were prepared and autoclaved separately, after which the ingredients were mixed and dispensed. The isolates were spot inoculated onto the plates and incubated at 37˚C for 24 - 48 hours. The development of a clear zone around the colonies indicated proteolytic activity (Pavlov *et al*., 2004; Bhowmik *et al*., 2015)

3.5.4 Lipase

Media containing 40 g/L tryptone soy agar (Merck, Germany) was prepared and supplemented with 1% Tween-80 (Sigma, Germany) to serve as a substrate. Isolates were spot inoculated onto plates and incubated at 37˚C. The formation of a turbid halo after 24 - 72 hours of incubation was an indication of lipase activity (Pavlov *et al*., 2004; Georgescu *et al*., 2016; Musa & Adebayo-Tayo, 2012).

3.5.5 Haemolysis

Blood agar containing 5% (v/v) sheep blood was used to determine the haemolytic ability of the bacterial isolates. Isolates were spot inoculated onto plates and incubated at 37°C. The lysis of red blood cells indicating varying degrees of haemolytic activity after 24 – 48 hours is considered to be an indication of haemolytic capabilities of the isolates (Mulamattathil *et al*., 2014)

Haemolytic activity is determined after the incubation on blood agar plates; the plate is inspected for signs of α-, β- or γ-haemolysis (Buxton, 2005). The blood content of the agar, combined with the high nutrient load makes it possible to support the growth of a wide variety of bacteria (Buxton, 2005). If the blood agar is discoloured or darkened after growth, the bacterial isolate has demonstrated α-haemolysis compared to when the blood agar plate develops a clear halo undergrowth, the isolate is β-haemolytic whereas if no noticeable change in the colour of the blood agar plate occurs, it is classified as γ-haemolysis (Buxton, 2005).

3.6 Antibiotic susceptibility

Cross-resistance to antibiotics was undertaken by using the Kirby-Bauer disk diffusion susceptibility test protocol (Hudzicki, J. 2009). Five antibiotics were used to assay for resistance to antibiotics. Antibiotics selected included: Vancomycin (30 µg), Kanamycin (30

µg), Trimethoprim (5 µg), Amoxicillin (10 µg) and Chloramphenicol (30 µg). Amoxicillin and Kanamycin are described as being predominantly bactericidal, effectively eradicating bacteria with up to 99% efficiency (Kohanski *et al*., 2007). In contrast, Trimethoprim, Tetracycline and Chloramphenicol are described as being predominantly bacteriostatic, meaning they inhibit bacterial growth without directly causing bacterial cell death (Kohanski *et al*., 2007). These antibiotics were selected due to their common use in human medicine and the documented instances of bacterial resistance in various water sources, as reported in previous studies (Kohanski *et al*., 2007; Horn *et al*., 2016). The five pharmaceuticals were selected as model drugs due to their high consumption rate in SA and from current studies (Netshithothole *et al*., 2024)

Pure bacterial colonies were selected and transferred into 2 ml sterile saline to prepare a bacterial suspension. The turbidity of the suspension was adjusted to a 0.5 McFarland standard (Hudzicki, J. 2009). 100 µl aliquots of each vortexed suspension were placed onto the Mueller-Hinton agar plates, thereafter a sterilized cell spreader was used to distribute the cells by streaking the spreader 3 times over the agar surface (Merck, Germany) (Hudzicki, J. 2009; Joseph *et al*., 2011). The plate rim was then swabbed to remove the excess liquid and left to dry with the lid slightly ajar (Hudzicki, J. 2009). The antibiotic disks were applied onto the agar-containing isolates using sterile tweezers and incubated at 37°C for 18 hours (Hudzicki, J. 2009; Joseph *et al*., 2011).

Table 3.2: Zone diameter interpretative standards according to the Clinical and Laboratory Standards Institute (2018).

Antibiotic	Abbreviation	Concentration	R (mm)	I (mm)	S (mm)
Amoxicillin	A10	10 µg	≤13	14 -17	≥18
Chloramphenicol	C30	30 µg	≤12	13 - 17	≥18
Kanamycin	K30	30 µg	≤13	14 - 17	≥18
Vancomycin	VA30	30 µg	≤14	15 - 16	≥17
Trimethoprim	T5	5 µg	≤15	16 - 18	≥19

R= resistant, I=Intermediate, S= Susceptible

Following the incubation of the micro-organisms and the antibiotic discs, the inhibition zone sizes were measured after 18 – 24 hours using a calliper and rounded off to the nearest millimetre (mm). Inhibition zones were divided into three groups classifying the isolates as resistant, susceptible, or intermediate by using the performance standards for antimicrobial disk susceptibility tests (CLSI, 2016, CLSI, 2018; McLain *et al*., 2016). Table 3.2 represents the Clinical and Laboratory Standards Institute (CLSI) for the determination of antibiotic susceptibility for the selected antibiotics. The results obtained were used to classify isolates

as being resistant, intermediate or susceptible to a specific antibiotic using the standards compiled by the Clinical and Laboratory Standards Institute (CLSI, 2016, CLSI, 2018).

3.7 DNA extraction

3.7.1 Environmental samples

For the freshwater and WWTP effluent samples, 100 ml of each sample was filtered through a 0.45 µM pore-sized filter (cellulose nitrate membranes, Whatman Laboratory Division, Maidstone, England) using a water pump (model Sartorius 16824). The membranes were cut into pieces with sterile scissors and aseptically placed onto sterile petri dishes for environmental bacterial DNA extraction. The sterile petri dishes containing the environmental bacterial DNA extraction membranes were stored at -20°C for further analysis.

DNA was extracted from the cut-up filter membranes in its entirety using the Nucleospin Soil DNA isolation kit (Macherey-Nagel) (Shi *et al*., 2018). The protocol was followed as stipulated by the manufacturer. The quality and quantity of extracted DNA was determined using the Nanodrop One (Thermo Fisher Scientific, USA) spectrophotometer. The extracted DNA was reported in ng/µl.

3.7.2 Pure bacterial cultures

DNA was extracted from isolated pure bacterial samples obtained using the Quick DNA fungal/Bacterial Mini prep kit (Zymo Research). The protocol was followed as stipulated by the manufacturer. Where applicable, the volume of the bacterial cell weight was adjusted to minimise the inhibition of optimal DNA extraction. The quality and quantity of the extracted DNA were determined using the Nanodrop One (Thermo Fisher Scientific, USA) spectrophotometer. The isolated DNA Integrity was assessed through conventional 1.5% (w/v) agarose gel electrophoresis (Das *et al*., 2012).

3.7.3 Microplastics and wood substrate

DNA was extracted from microplastics and wood samples using the Nucleospin Soil DNA isolation kit (Macherey-Nagel) (Onda & Sharief, 2021). When preparing the samples for DNA extraction, 12 particles (microplastic and wood) were placed aseptically into the NucleoSpin Bead Tube Type A (Macherey-Nagel). The protocol was further followed as stipulated by the manufacturer. Where applicable, the volume of reagents for microplastic samples was adjusted due to the small sample size to minimise the inhibition of optimal DNA extracted. The quality and quantity of extracted DNA was determined using the Nanodrop One (Thermo

Fisher Scientific, USA) spectrophotometer and reported as ng/µL. The amount used for each substrate remained constant to ensure that the different substrates could be compared against each other. The DNA yield was measured per extraction volume

3.7.4. Electrophoresis of polymerase chain reaction products

Amplifications were performed using the Applied Biosystems A37028 MiniAmp Thermal Cycler 96-Well ABI PCR Machine. The reactions were prepared in 25 µL, 1 µL of the template DNA (~ 21 – 340 ng), 10 µM of each primer (Forward (27F) and reverse (1492R) primers), 12.5 µl PCR master mix (Thermo Fischer Scientific, USA), and 8.5 µl nuclease-free water within a laminar flow (Bioflow I) cabinet. The forward primer (27F) binds to the beginning of the 16S rRNA region (5'-AGAGTTTGATCMTGGCTCAG-3') of a gene, and in doing so initiates amplification, whereas the reverse primer (1492R) targets the end portion of the 16S rRNA region (5'-TACGGYTACCTTGTTACGACTT-3') and thereby completing amplification of the gene (Frank *et al*., 2008). Thermal cyclic conditions include 95°C for 5 minutes (the initial denaturation), followed by 30 cycles of 95°C for 1 minute (denaturation), 54°C for 30 seconds (annealing), 72°C for 60 seconds and lastly 72°C for 5 minutes (elongation) (Nascimento *et al*., 2024; Li *et al*., 2009).

The PCR products were separated by electrophoresis on 1.5% (w/v) agarose gel on a horizontal Pharmacia Biotech equipment system (model Hoefer HE 99X; Amersham Pharmacia Biotech, Sweden) at 80 V for 45 minutes using 1X TAE buffer (40 mM Tris, 1 mM EDTA and 20 mM glacial acetic acid combined with a pH of 8.0 (Lorenz, 2012, Riet *et al*., 2017; Mulamattathil *et al*., 2014). The agarose gel contained 10 µl ethidium bromide (Biorad, UK). A mixture of 5 µl PCR amplification product and 2 µl 6x orange loading dye (Thermo Fischer Scientific, USA) was loaded into each well of the gel. Each gel contained 1000 bp and 100 bp DNA molecular markers (O'GeneRuler, Thermo Fisher Scientific, USA) to compare the PCR amplification fragment size (Lorenz, 2012; Riet *et al*., 2017). The gel images were captured using the Chemidoc MP imaging system (Bio-Ras, USA) using Image Lab software (version 4.0.1). The PCR products were supplied by Inqaba Biotechnological Industries Pty Ltd, Pretoria, SA. The successful PCR products were then stored at - 4°C.

3.8 DNA sequencing and analysis

Amplified DNA fragments from the PCR experiment were used for sequencing studies. The first clean-up was done using the AMPure XP PCR purification process (Beckman Coulter Genomics, USA) (Singer *et al*., 2016). Firstly, the AMPure XP bottle was shaken to redistribute the magnetic particles, 36µl of magnetic beads were added to the remaining 20 µl PCR product

into a reaction plate and thoroughly mixed by pipetting the mixture up and down a few times and incubate at room temperature for 5 minutes (Singer *et al*., 2016; Chen *et al*., 2021). Thereafter, the reaction plate is placed onto a 96 super-magnet plate for 2 minutes to separate the magnetic beads from the mixture, the clear solution was then discarded, leaving behind the magnetic beads and the supernatant (Singer *et al*., 2016). Within the reaction plate, 200 µl of 80% ethanol was distributed into each well and incubated for 30 seconds at room temperature, the ethanol was removed, and the step was repeated twice and let dry for 10 minutes (Singer *et al*., 2016). Thereafter the reaction plate was removed from the super magnet, 40 µl of elution buffer was added to each well and pipette mixed 10 times and incubated for 2 minutes (Singer *et al*., 2016). Lastly, the reaction plate was placed onto the super magnet for 2 minutes to separate the beads from the solution, the elution was then transferred to a new plate (Singer *et al*., 2016).

Sequencing of the Purified PCR product was performed using the BigDye Terminator v3.1 cycle sequence kit (Thermofisher, USA). The ready reaction mixes and 27F and 1492R primers were diluted (1:10 dilution). A master mix contained 4 µl of ready reaction mix, 2 µl 5x of BigDye sequencing buffer, 3.2 pmol forward or reverse primer, 2 µl purified PCR template (10 – 50 ng) and 16 µl of nuclease-free water to add up to 25 µl. Thermal cyclic conditions include 96°C for 1 minute (the initial denaturation), followed by 25 cycles of 96°C for 10 seconds (denaturation), 50°C for 5 seconds (annealing), 60°C for 4 minutes (elongation) and placed immediately on ice (Xiong *et al*., 2006).

The second clean-up was performed using the Agencourt CleanSeq magnetic beads method (Fujii *et al*., 2022). Once the CleanSeq reagent is fully suspended, 10 µl was added to each sample, 62 µl of 85% ethanol was also added and mixed several times. The sample plate was then placed onto the magnetic stand for 5 minutes, the clear solution was discarded and 100 µl of 85% ethanol was dispensed into each well, after 30 seconds, the ethanol was removed and repeated (Fujii *et al*., 2022). The samples were then left to air dry for 10 minutes, thereafter 15 µl of elution buffer was added to each well and incubated for 5 minutes at room temperature. The magnetic beads were allowed to separate for 5 minutes, 12 µl of the solution was transferred to a new plate and placed into the sequencer (Fujii *et al*., 2022).

Sanger sequencing was performed on the pure bacterial isolates, in-house, using SeqStudio Genetic Analyzer (Thermo Fischer Scientific). Geospiza Finch TV (version 1.4) software was used to view chromatograms, and BLAST (Basic Local Alignment Search Tool) searches were used to determine the identity of the amplified sequences (http://www.ncbi.nlm.nih.gov/BLAST).

Metabarcoding of the environmental samples, such as the plastisphere, was performed using the Illumina Miseq platform (in-house) via the amplification of the V3-V4 hypervariable region in the 16S rRNA gene. The partial 16S rRNA gene libraries were quantified using a Qubit 3.0 fluorometer (Thermo Fisher Scientific, USA), normalized, pooled, and denatured in 0.2 N NaOH. Denatured libraries, along with 10% PhiX sequencing control, were then loaded onto the MiSeq flow cell using the MiSeq v3 reagent kit for a paired-end (2 × 300 bp) sequencing run.

Next-generation sequencing on the Illumina Miseq analyser start with DNA fragmentation, DNA end-repair, adapter ligation, surface attachment and *in-situ* amplification (Hu *et al*., 2021). Illumina Miseq analyser uses a sequencing-by-synthesis approach in which all four nucleotides are added simultaneously into oligo-primed cluster fragments in flow-cell channels along with DNA polymerase and presents a 95% raw base accuracy (Zhang *et al*., 2011). The overall sequence read output, in combination with the ability to multiplex samples, makes MiSeq amplicon sequencing cost-effective. However, technical difficulties may occur during library preparation, resulting in low read quality or low clustering and read recovery rate (Chen *et al*., 2021). PhiX (short DNA fragments derived from a bacteriophage genome) is added upon sequencing to optimize sequencing quality (Chen *et al*., 2021).

3.9 Data analysis

Microsoft Excel was used to calculate averages, R^2 values, standard deviation, and efficiency of the DNA quality and quantity. Pure bacterial isolates were identified by using the Maximum Likelihood method and the Tamura-Nei model due to the robustness and estimation of evolutionary change (Lin *et al*., 2013; Qiang *et al*., 2021). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the analysed taxa (Felsenstein, 1985, Tamura *et al.,* 2021). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown on the branches of the tree. The initial tree was obtained by applying the Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model and then selecting the topology with superior log likelihood value (Felsenstein, 1985, Tamura *et al.,* 2021). Evolutionary analyses were conducted in MEGA11.

3.9.1. Phylogenetic diversity analysis

Alpha diversity indices were calculated at the genus level for richness (Chao1 estimator), diversity and evenness (Shannon index), and species dominance (Simpson index) were calculated through the iNext package in R. Analysis of variance (ANOVA) was performed for each index to identify differences between the substrates (Pinto *et al*., 2019; Tillier & Collins,

2000). All analyses were performed in triplicate, and the experimental values were expressed as the mean ± standard deviation (s.d.). Bacterial community structure and composition were compared using the non-metric multidimensional scaling (nMDS) analysis employing the Jaccard similarity matrix within the computing environment, R Studio, to visually represent the similarity of the community composition between the samples (Pinto *et al*., 2019). Non-metric multidimensional scaling is efficient at identifying underlying gradients and at representing relationships (Ramette, A. 2007).

3.9.2. Bacterial community composition

Data obtained from the MiSeq sequencing platform was processed for downstream identification in R. Errors in the Illumina sequences amplicon data were addressed by quality filtering and construction of Operational Taxonomic Units (OTU), thereby filtering and reducing the error rate through use of the DADA2 package, consisting of the merge of pair-end reads after denoising and removal of the dereplicated reads, (Callahan *et al*., 2016; Barnes *et al*., 2020). Filtering and trimming steps were applied with the following settings: truncLen=c (250, 250), minLen = c (220, 220), maxEE=c (10, 10), maxN = 0, rm. phix=TRUE to the forward and reverse reads separately. Thereafter, the learnErrors and dada functions were applied to the forward and reverse reads independently and merged through the mergePair function. An initial sequence table was constructed before chimaeras were identified through the removeBimeraDenovo function (Barnes *et al*., 2020; Flegontova *et al.*, 2023). The denoised reads were assigned taxonomy by the Silva 16S rRNA database (138.1) by the assignTaxonomy function (McLaren & Callahan, 2021). At various points, the rds data structures were saved, including taxonomy tables and sequences. Downstream statistical analyses were carried out using phyloseq, a phylogenetic sequence display tool (Callahan *et al.,* 2016; McMurdie & Holmes, 2012; McMurdie & Holmes, 2013; McMurdie & Holmes, 2019).

Chapter 4 : Results

This chapter summarises and interprets the results based on the aims and objectives outlined in Chapter 1. This chapter provides the interpretation of the results obtained from the microcosm studies conducted in 2022 to characterise and analyse microbial colonisation on microplastics and wood substrates. Sections 4.1 to 4.3 provide behavioural information about the micro-organism attachment towards microplastics. Section 4.4 highlights the DNA extracted from the microplastics and wood substrate, whereas section 4.5 highlights the microbial consortia detected on the substrates versus filtrate water pre-incubation. Section 4.6 focuses on the characterisation of bacterial isolates obtained from the microplastics, and section 4.7 explores the prevalence of ARB and water to gain insight into substrates acting as possible vectors of ARG in freshwater environments. The chapter further discusses potentially pathogenic bacterial identification in sections 4.8 to 4.10. Lastly, section 4.11 explores the microbial consortia (based on NGS) detected on the substrates and water sources post-incubation.

4.1. Microbial colonisation on substrates

Simulated freshwater environments were achieved through the incubation of sterile microplastics and wood particles in a mixture of river water and WWTP effluent. Scanning electron microscopy was performed at the commencement and culmination of the experiment to supply pictures of the microbial biofilm growth on the various substrates. Figure 4.1 indicates that microbial cells were absent on all the substrates after sterilisation and before incubation at the start of the experiment. A clear distinction between the surfaces of the various substrates was observed pre-incubation. The wood substrate had compartments, compared to the uneven surface structure of HDPE. The nylon particle is seen to have an uneven surface combined with compartments. LDPE and HDPS particles presented a smooth surface before incubation.

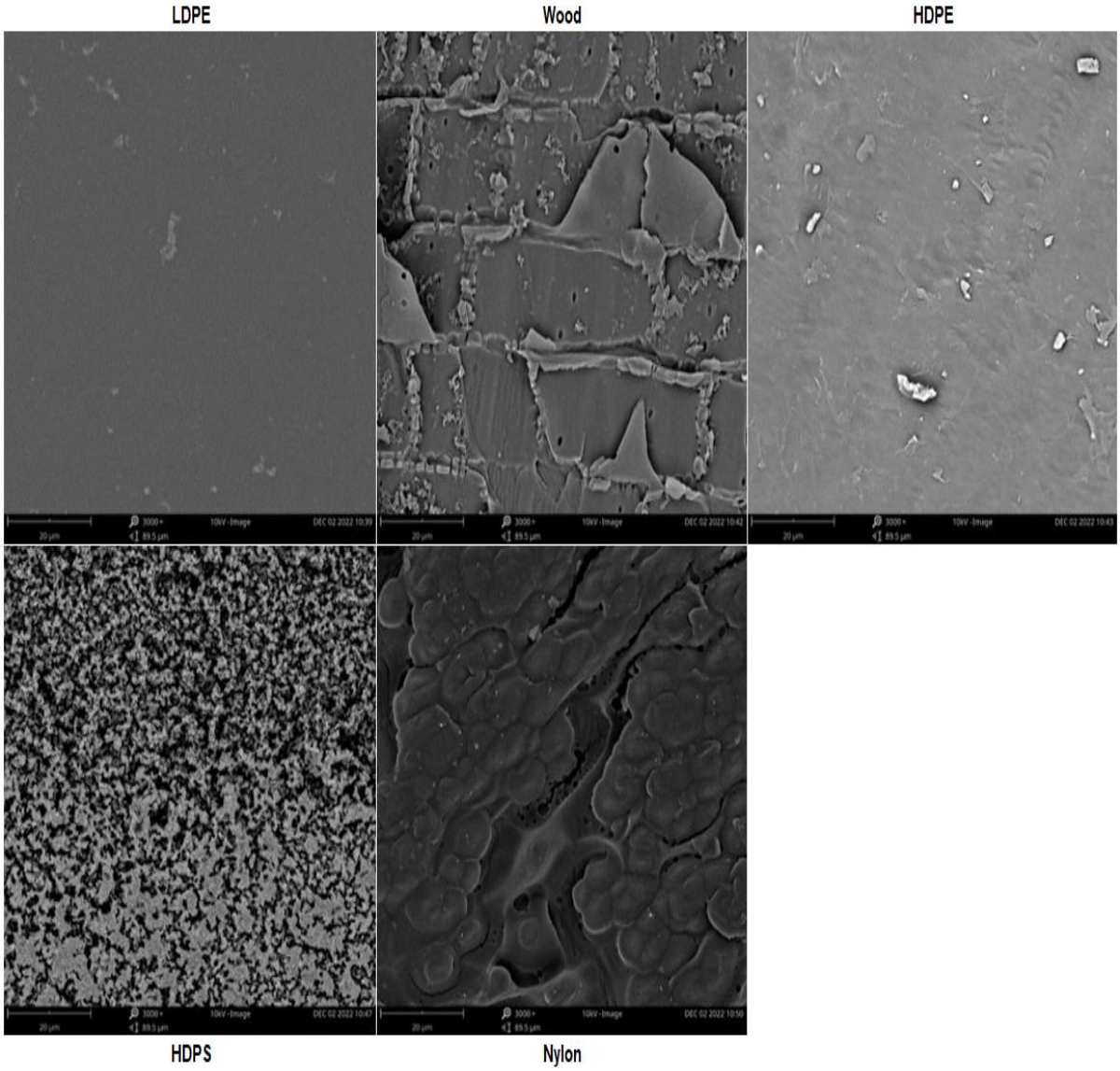


Figure 4.1: Scanning electron microscopy images of microplastics and wood substrates pre-incubation (Day 0).

4.2. Microbial colonisation on wastewater treatment plant effluent microcosm substrate

The microplastics were incubated in sterilised river water and WWTP effluent. Due to the surface composition of the various particles, the microbial attachment was higher in the crevices compared to the whole surface of the substrate (Figure 4.2). The composition of the substrate surface was found to undergo significant changes after 28 days. The SEM of the HDPS indicated that microbial cell attachment occurred in the crevices and grooves. The cluster of cells composed of rod and round-shaped cells, along with spherical cells, were covered by a film layer, indicating the presence of a biofilm. The yellow circle indicates the different morphological structures. Low-density polyethylene indicated that biofilm formation

occurred due to the presence of a film; the bacterial cells could be described as rod-shaped, and cell clusters were also seen. Very few morphological identifications could be made due to the slime barrier created by the microbial assemblages on HDPE. The few microbial cells that could be seen are rod-shaped. The microbial assemblage on nylon could be described as a mixture of rod, round, and spiral-shaped cells. The wood substrate structure is complex, and the bacterial assemblages were more advanced due to the uneven surfaces, crevices and folds combined with the nature of organic matter to break down. The clustering of cells can be seen along with the web-like attachment of cells.

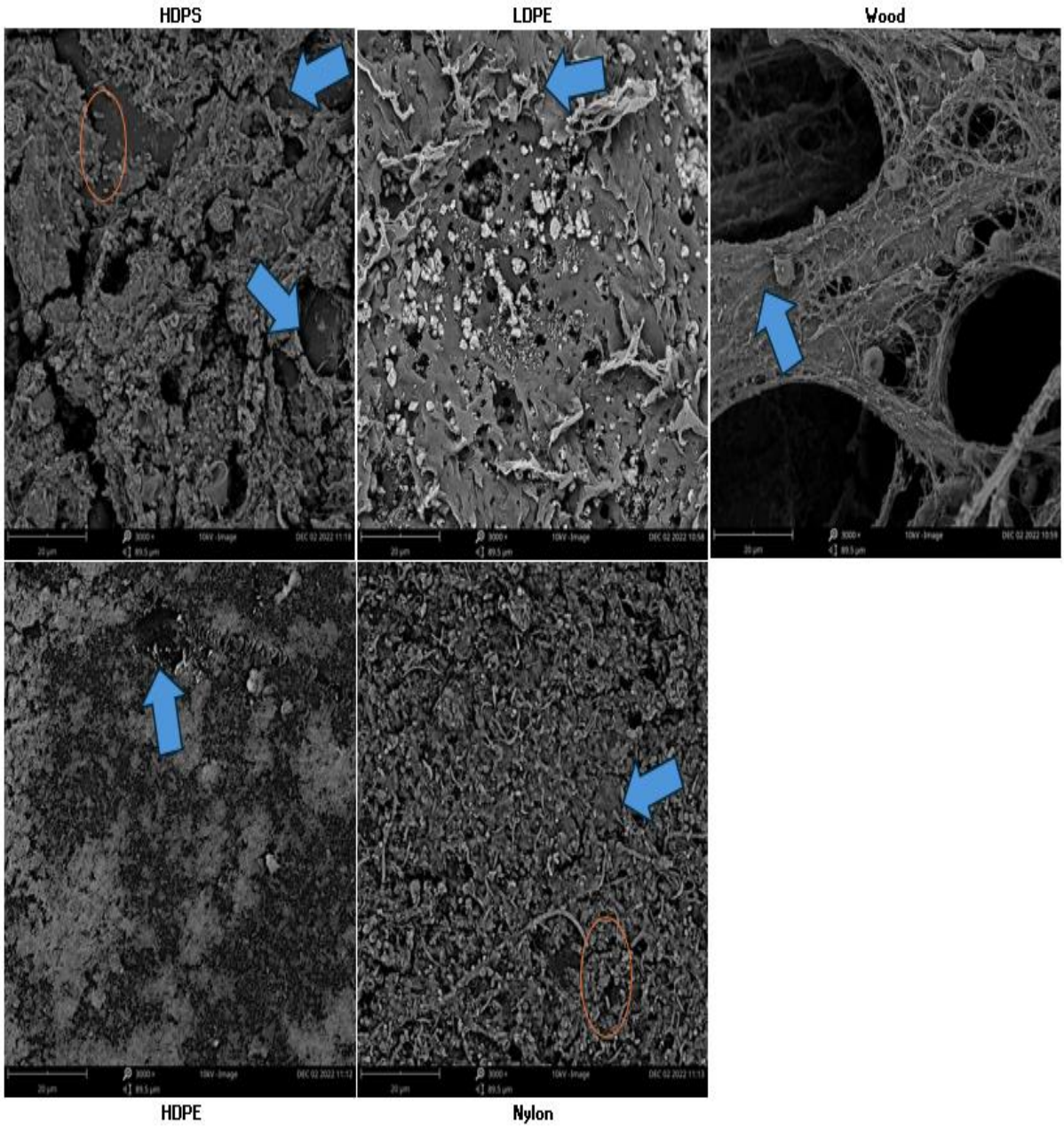


Figure 4.2: Microplastic particles isolated from the combined sterilised river and wastewater treatment plant effluent microcosms showing microbial colonisation on the surface on day 28. Biofilm formation is indicated by the arrows.

4.3. Microbial colonisation within the positive control microcosm

The unsterilised WWTP effluent with the different types of microplastics served as the positive control microcosm. Through investigating the microbial attachment on the LDPE surface, rod-shaped bacteria along with a partially produced film, and clustering of microbial cells within the crevices on the surface were observed (Figure 4.3). Scanning electron microscopy of HDPE in the positive control microcosm provided an inaccurate picture; the overpopulation of microbial cells produced a slime barrier that prevented the SEM from producing a clear picture of microbial cell colonization and prevented comparison between the substrates.

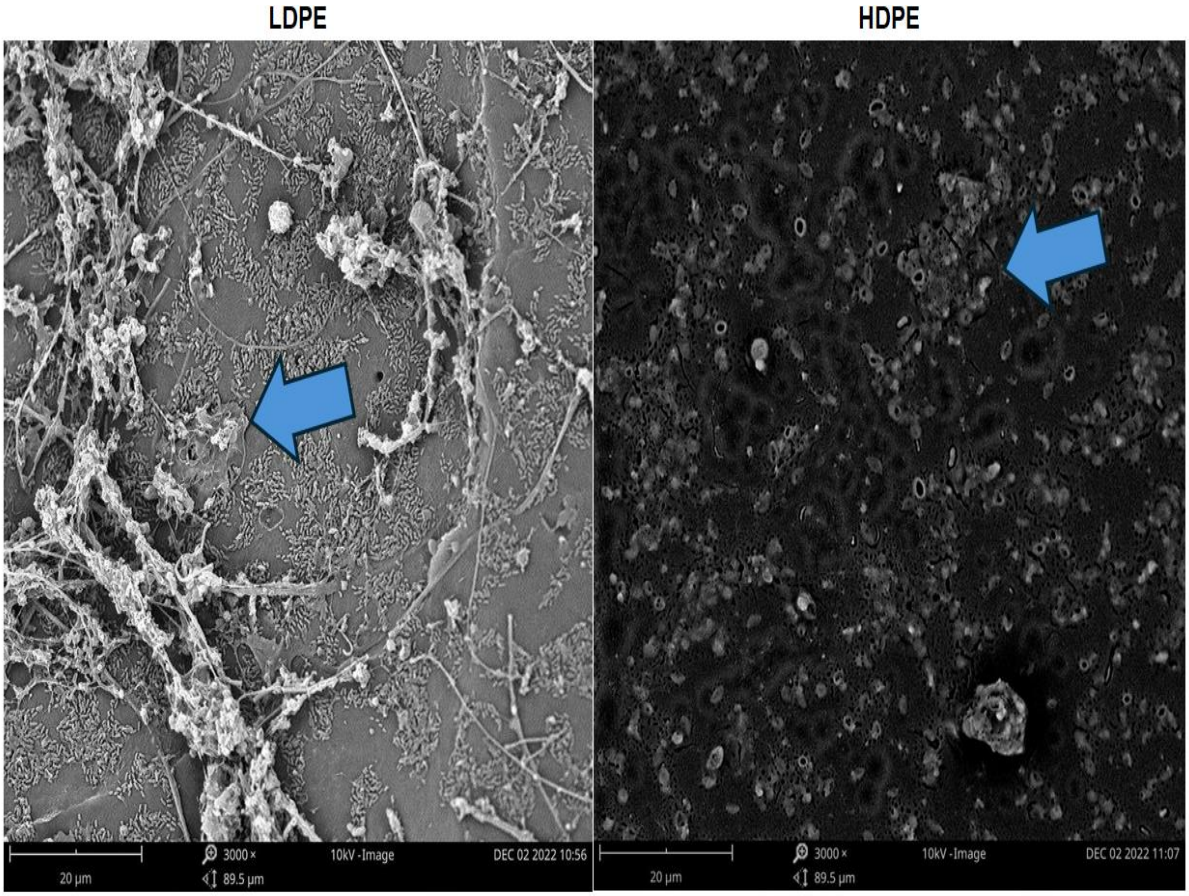


Figure 4.3: Scanning electron microscopy of micro-plastics retrieved from the positive control microcosm on day 28.

4.4. Microbial colonisation comparison

Figure 4.4 indicates the microbial colonisation of a sterilised HDPS particle incubated for 28 days as seen on SEM under 20 µM magnification. On the HDPS sample, microbial attachment was seen to occur within the grooves, pits, and ledges of the sample. No bacterial attachment was observed on Day 0 compared to the biofilm formation and attachment to the substrate surface on Day 28. A plethora of bacterial cells were seen due to a difference in morphology,

such as filaments, bacilli-shaped, and spiral-shaped cells. However, it cannot be deduced whether there was a change in microbial composition on the substrate due to observations made on different microplastic samples of the same polymer type on other days.

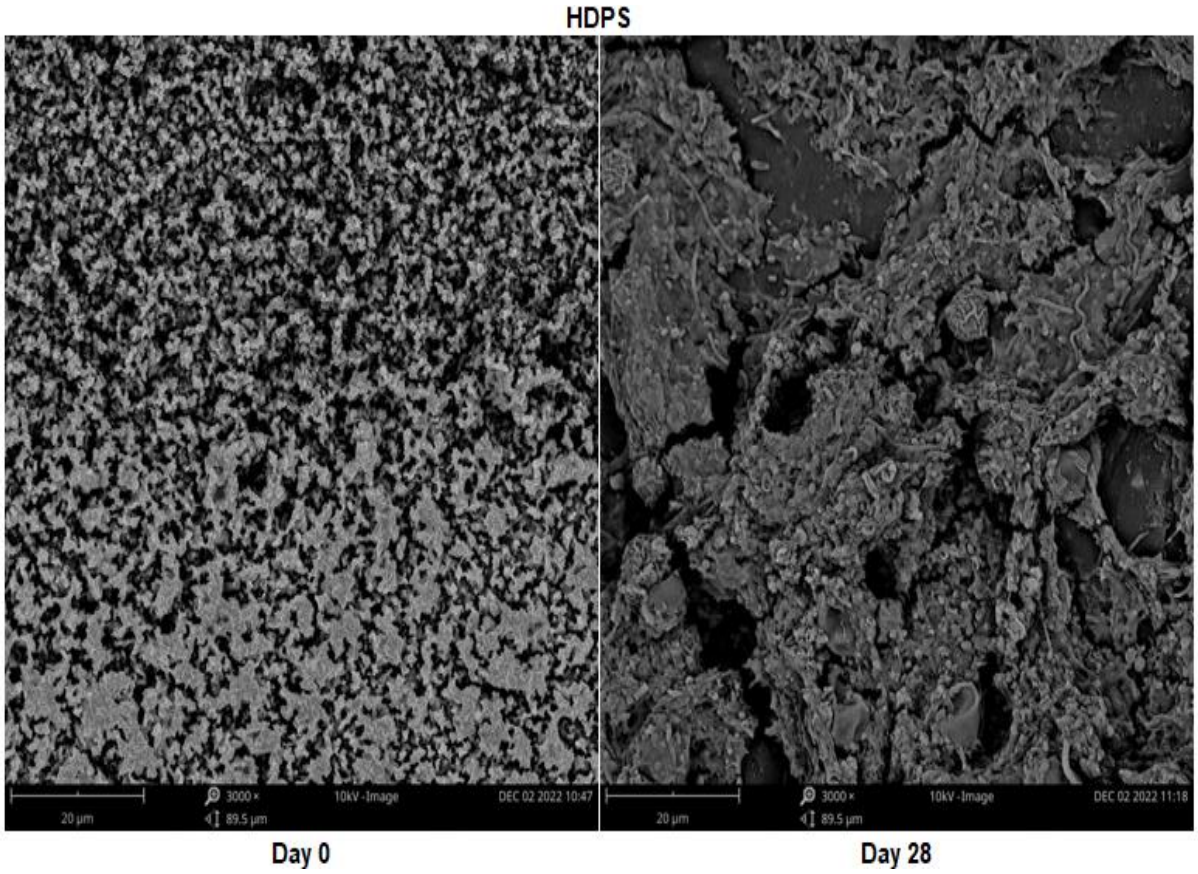


Figure 4.4: Scanning electron microscopy comparison between microbial colonisation on high-density polystyrene on different days from the wastewater treatment plant effluent microcosm.

Figure 4.5 Indicates the biofilm attachment on nylon microplastic after 28 incubation days under 20 µM magnification. Different microbial assemblages consisting of different bacterial species can be seen on the nylon microplastic on day 28. The surface structure aided in microbial attachment, making bacterial attachment to the pits and grooves possible. Microbial diversity could also be seen on the nylon substrate. Day 0 presented a sterilised nylon pellet compared to the post-incubation nylon pellet (day 28). It cannot be said that microbial composition changed due to the different sampling days and different particles from the same polymer.

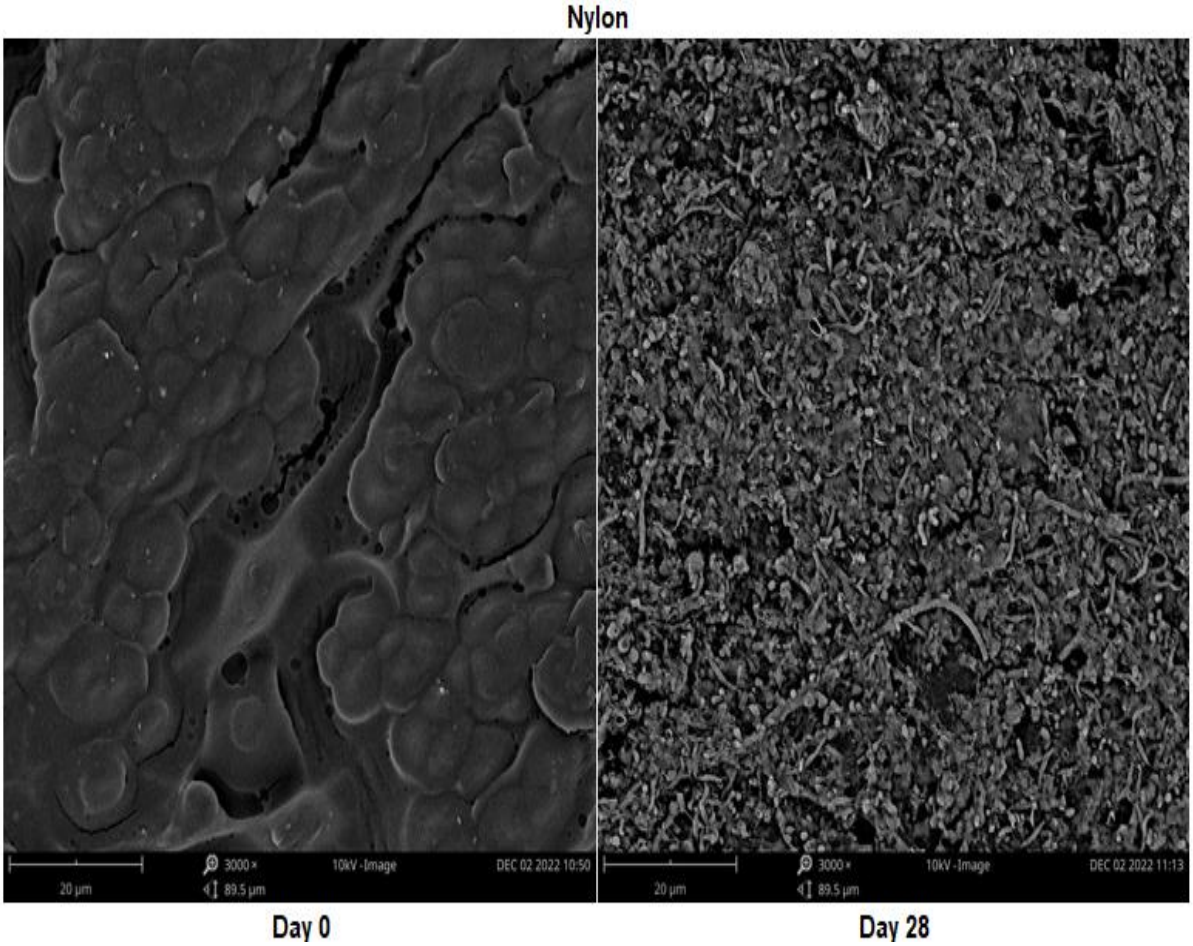


Figure 4.5: Scanning electron microscopy comparison between microbial colonisation on nylon on different days from the wastewater treatment plant effluent microcosm.

Figure 4.6 indicates the biofilm attachment on the Wood substrate after 28 incubation days under 20 µM magnification. On day 0, the wood substrates were seen to have grooves, ledges, and uneven surfaces, but bacterial colonisation was absent due to the pre-incubation sterilisation of the substrate and the immediate fixation for SEM. Day 28 indicated the microbial colonisation of the wood substrate, the uneven surface combined with grooves and ledges provided sites for attachment of microbial community. Rod and filamentous-shaped microbes were observed to be present on the surface of the wood particles.

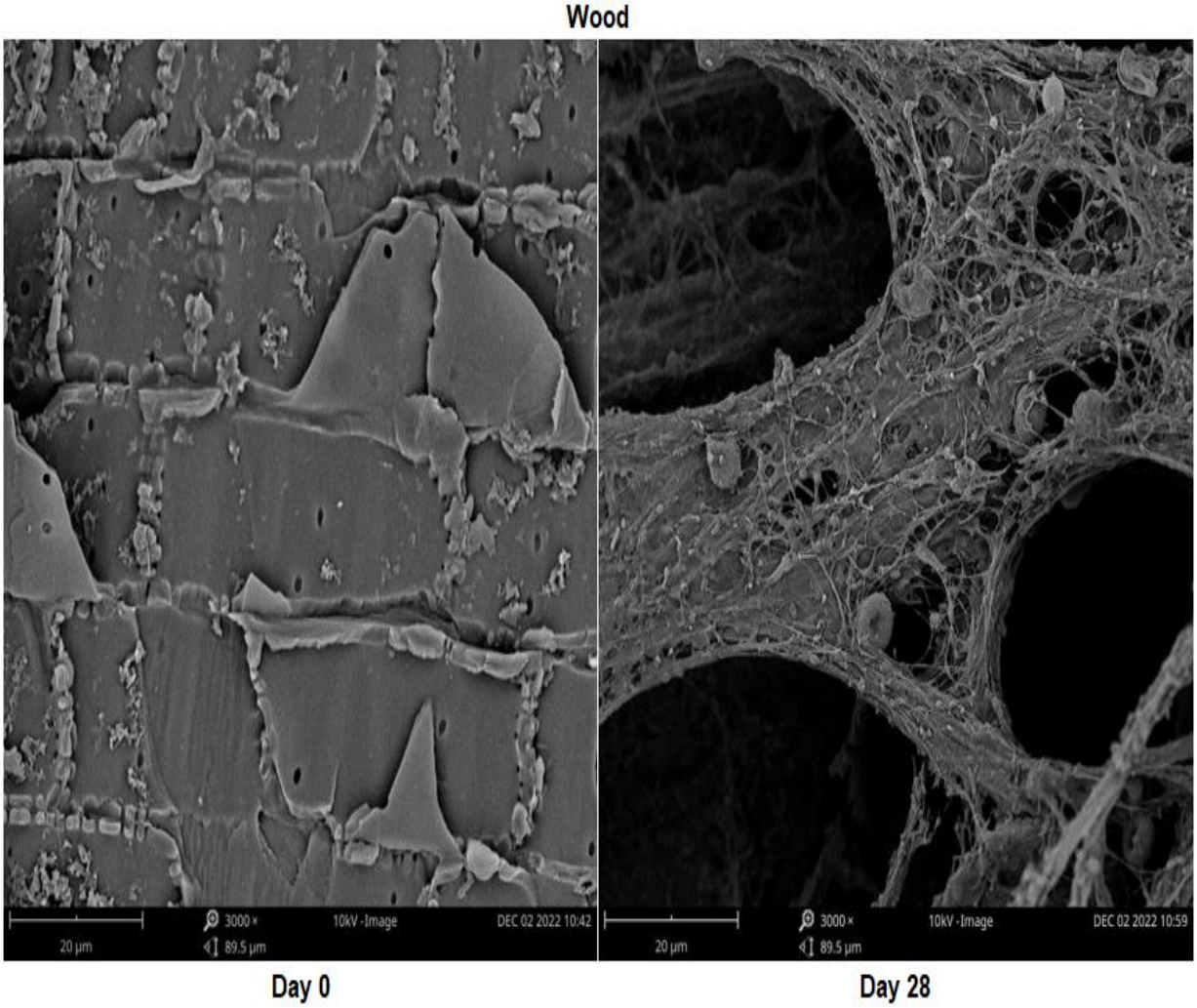


Figure 4.6: Scanning electron microscopy comparison between microbial colonisation on the wood substrate on different days from the simulated freshwater microcosms.

4.5. DNA yield quantity and quality

The DNA concentrations of the samples varied between the sample types (substrates and water). The filtrate water samples ranged from 11.4 ng/µl to 117.1 ng/µl with a mean average of 52.41 ng/µl. The DNA concentrations from the microplastic samples obtained from the microcosms that were used for downstream application ranged from 16.4 ng/µl to 81.1 ng/µl with a mean average of 41.9 ng/µl. The concentrations of the wood samples ranged from 9.4 ng/µl to 145 ng/µl with a mean average of 84.4 ng/µl ± 55.97 ng/µl. The concentrations of the nylon samples ranged between 28.4 ng/µl and 59.5 ng/µl with a mean average of 48.52 ± 13.79 ng/µl. The concentration of the HDPS samples ranged from 22.8 ng/µl and 69.8 ng/µl with a mean average of 43.97 ng/µl ± 22.89 ng/µl. The concentrations of LDPE samples ranged from 21.8 ng/µl and 46.5 ng/µl with a mean average of 33.97 ng/µl ± 10.4 ng/µl. The concentrations of the HDPE samples ranged from 16.4 ng/µl to 25.7ng/µl with a mean average of 20.02 ng/µl ± 4.21 ng/µl. River water sample concentrations ranged from 11.4 ng/µl to 60.3

ng/µl with a mean average of 31.13 ng/µl ± 19.93 ng/µl per 100 ml. The wastewater sample concentrations ranged from 76.1 ng/µl to 117.1 ng/µl per 100 ml with a mean average of 94.96 ng/µl ± 20.69 ng/µl (see Figure 4.7).

The DNA quality of the microplastics and wood substrates retrieved from the microcosms was relatively similar, with an absorbance ratio (A_{260}/A_{280}) of 1.84 to 1.88, and the water samples ranged from 1.83 to 1.84. The DNA extractions led to the successful downstream application to yield the presented results.

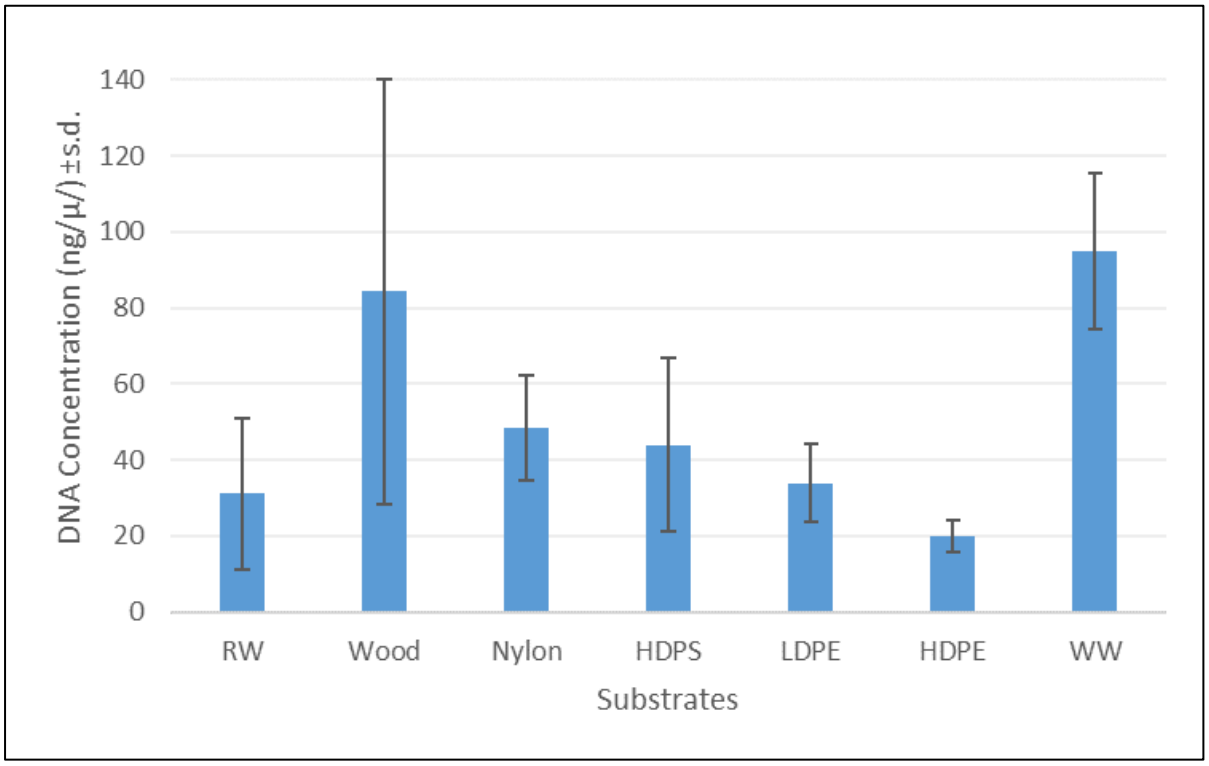


Figure 4.7: Mean average of DNA concentrations (ng/µl ± standard deviation (s.d.)) of the sample types: Wood from the microcosm, river water filtrate direct from the environment (RW), wastewater effluent from the environment (WW), nylon, HDPE, LDPE and HDPS collected from the microcosm.

4.6. Gram staining and virulence testing

Table 4.1 indicates the hemolytic activity and the presence of several extracellular enzymes associated with virulence factors. Virulence testing was conducted on the pure bacterial isolates obtained through the plastic plating and streak plating method. The newly assigned number will be used further throughout for simplification purposes, as seen in Table 4.2.

A total of 877 pure colonies were isolated (refer to the method section). A value of 0.5 - 1 was awarded to each isolate, depending on the type of hemolytic reaction the bacterial isolates presented. β-Haemolysis was awarded a value of 1, whereas α-haemolysis was awarded a value of 0.5 due to the partial breakdown of the red blood cells (Almwafy, 2020). If the bacterial

isolate possessed the ability to break down proteins, catalyse the hydrolysis of fats as well as degrade DNA, a value of 1 was awarded to the corresponding enzymatic tests (see Table 4.2). In this table, the number of bacterial isolates that tested positive for the different virulence tests conducted ranged between 3.5 and 4, through which the total bacteria isolated were lessened to 51 usable strains for further identification.

Out of 877 isolates, 481 appeared to present ɣ-haemolysis, α-haemolysis appeared 204 times, and β-haemolysis occurred 183 times; six isolates had no growth on the blood agar plates. Figure 4.8 indicates the percentage of isolates that tested positive for the production of extracellular enzymes. Of the 877 isolates examined, 49 % tested positive for haemolysins, 32% for Proteinase, 27% for DNase and lastly 38% for the presence of Lipase (see Figure 4.8). Haemolysins and lipase were the most prevalent extracellular enzyme produced, whilst DNase was the least produced.

Gram staining was performed on the 51 selected microbial isolates obtained from the completed virulence testing for further identification. Gram-positive isolates were identified by the blue colouring of the cells due to the thicker peptidoglycan layer, which results in the safranin dye partially penetrating the cell membrane compared to the Gram-negative bacteria that stained red (Figure 4.9). A total of 14 out of 51 bacterial isolates presented Gram-positive compared to the 37 Gram-negative isolates, as seen in Figure 4.10.

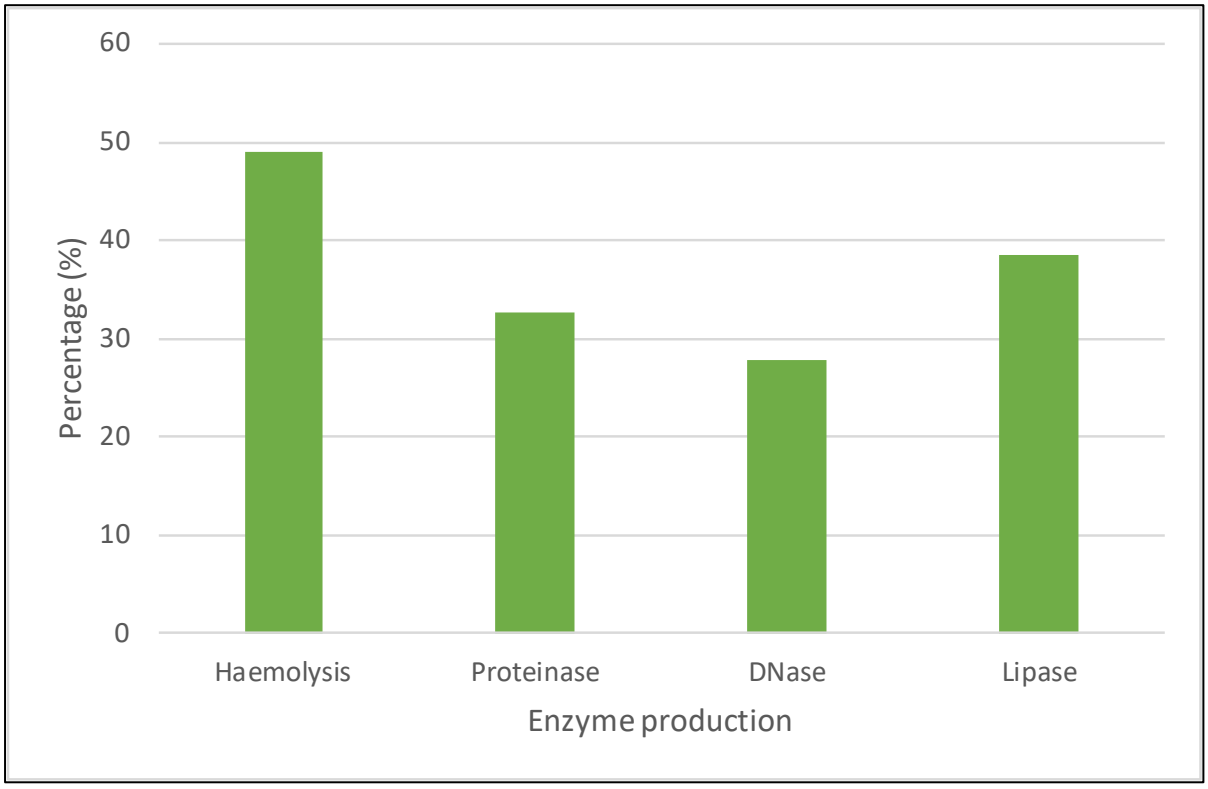


Figure 4.8: Percentage of extracellular enzyme production.

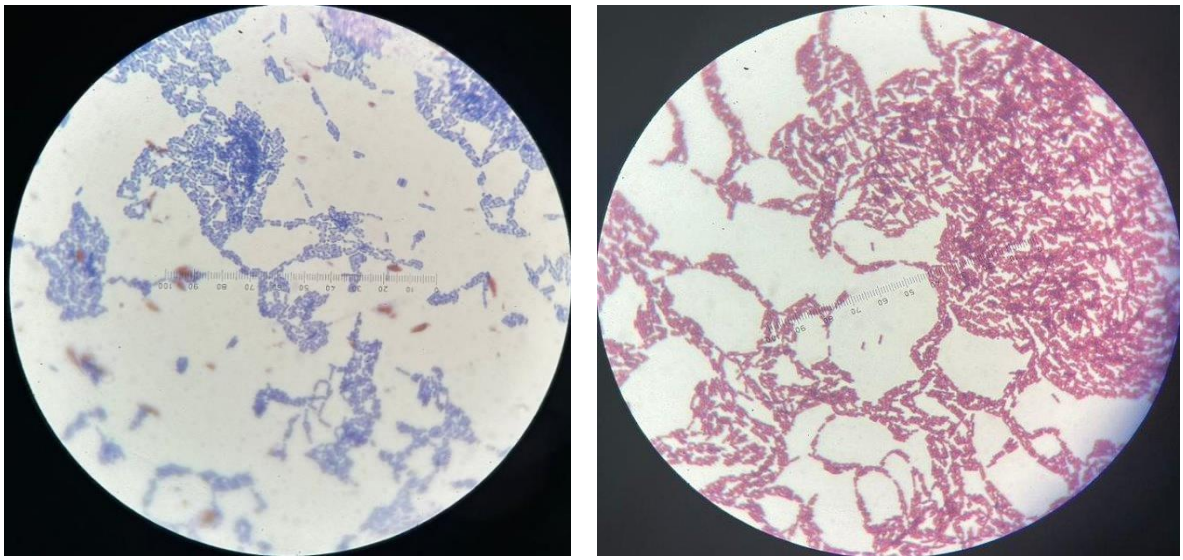


Figure 4.9: Illustration of the difference in gram staining results, performed on isolates under a microscope at 1000x magnification.

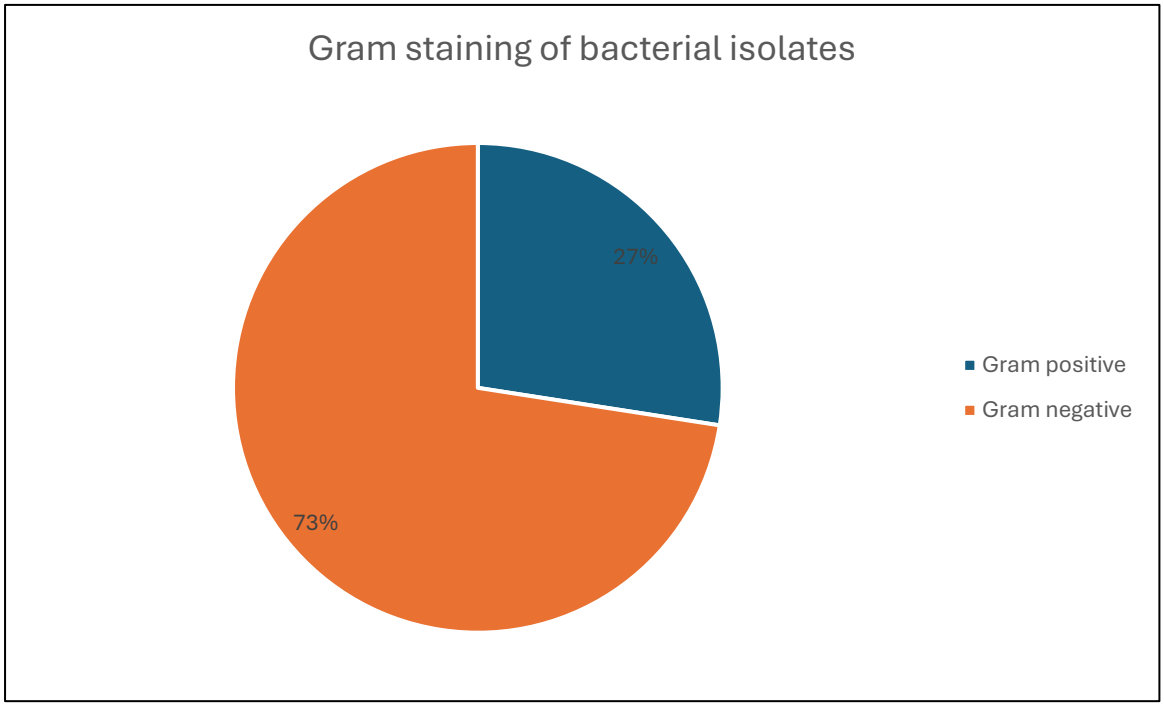


Figure 4.10: Gram staining of microbial colonies after virulence testing for further identification.

Table 4.1: Extracellular enzymatic testing on bacterial isolates.

Isolate number	Newly assigned number	Haemolysis	Proteinase	DNase	Lipase	Sum
131	1	1	1	1	1	4
147	2	0.5	1	1	1	3.5
158	3	0.5	1	1	1	3.5
170	4	1	1	1	1	4
198	5	1	1	1	1	4
199	6	1	1	1	1	4
232	7	0.5	1	1	1	3.5
249	8	1	1	1	1	4
261	9	1	1	1	1	4
264	10	1	1	1	1	4
285	11	0.5	1	1	1	3.5
342	12	0.5	1	1	1	3.5
358	13	0.5	1	1	1	3.5
359	14	0.5	1	1	1	3.5
364	15	0.5	1	1	1	3.5
365	16	0.5	1	1	1	3.5
374	17	0.5	1	1	1	3.5
381	18	1	1	1	1	4
420	19	0.5	1	1	1	3.5
433	20	0.5	1	1	1	3.5
496	21	0.5	1	1	1	3.5
507	22	0.5	1	1	1	3.5
508	23	0.5	1	1	1	3.5
509	24	0.5	1	1	1	3.5
510	25	0.5	1	1	1	3.5
511	26	0.5	1	1	1	3.5
555	27	0.5	1	1	1	3.5
564	28	0.5	1	1	1	3.5
645	29	0.5	1	1	1	3.5
666	30	1	1	1	1	4
682	31	1	1	1	1	4
695	32	0.5	1	1	1	3.5
696	33	0.5	1	1	1	3.5
709	34	1	1	1	1	4
722	35	1	1	1	1	4
725	36	1	1	1	1	4
729	37	1	1	1	1	4
730	38	1	1	1	1	4
731	39	1	1	1	1	4
733	40	1	1	1	1	4
747	41	0.5	1	1	1	3.5
765	42	1	1	1	1	4
767	43	1	1	1	1	4
774	44	1	1	1	1	4
779	45	1	1	1	1	4
816	46	0.5	1	1	1	3.5
818	47	1	1	1	1	4
821	48	1	1	1	1	4
822	49	1	1	1	1	4
824	50	1	1	1	1	4
875	51	1	1	1	1	4

4.7. Antibiotic profiles

Five (5) antibiotics that are commonly used for treatment purposes (van Driel *et al*., 2022), were used to determine the susceptibility, resistance, and intermediate resistance of the purified isolates. Antibiotic susceptibility testing was performed on the 51 bacterial isolates obtained from the virulence testing through the use of the Kirby-Bauer disc diffusion method (Table 4.2). The susceptibility of the isolates to the antibiotics was examined according to the guidelines of the CLSI (CLSI, 2016, CLSI, 2018). The selected antibiotics were placed onto Mueller-Hinton agar plates. The antibiotics included VA30, A10, K30, C30 and T5. A value of 1 was awarded towards bacterial isolates that presented resistance, a value of 0.5 was awarded to intermediate resistance and susceptible bacteria were awarded 0 (see Table 3.2).

Table 4.2 represents the percentage of results obtained after AST was performed. The following antibiotic resistance patterns were observed. 41.1% of the microbial isolates were resistant to VA30; 78.4% were resistant to A10; 13.8% to K30; 11.7% to C30 and 64.7% were resistant to T5

Table 4.2: Antibiotic resistance profiles of unknown isolates

Isolate number	*VA30*	*A10*	*K30*	*C30*	*T5*
1	S	R	S	S	R
2	S	R	S	S	R
3	R	R	S	S	I
4	S	R	S	S	R
5	S	R	S	S	R
6	S	R	S	S	R
7	S	I	S	S	S
8	S	R	S	S	R
9	S	R	S	S	R
10	S	R	I	S	R
11	S	R	S	S	R
12	S	R	I	S	R
13	R	R	I	S	I
14	R	R	I	S	R
15	R	R	I	S	I
16	R	R	I	R	R
17	R	R	S	R	R
18	S	R	S	S	R
19	S	I	S	S	R
20	R	R	S	I	I
21	I	S	S	S	R
22	S	S	S	S	R
23	R	R	R	R	R
24	S	R	S	S	R
25	S	R	S	S	S
26	R	S	S	S	S
27	S	R	R	R	R
28	S	S	S	S	R
29	R	R	R	I	R
30	S	S	S	S	S
31	S	S	S	S	S
32	R	S	S	S	R
33	R	I	R	S	R
34	S	R	S	S	R
35	S	R	S	S	R
36	R	R	R	S	R
37	S	R	I	I	R
38	R	R	I	S	I
39	R	R	I	S	I
40	R	R	I	S	I
41	R	R	S	S	S
42	R	R	S	R	R
43	R	R	S	S	S
44	I	R	R	I	R
45	R	S	S	S	S
46	R	R	R	I	R
47	I	R	S	S	S
48	I	R	S	S	S
49	I	R	S	S	S
50	S	R	S	R	R
51	S	R	S	S	R
Resistance percentage (%)	41.1	78.4	13.7	11.7	64.7

R= Resistant, I= Intermediate resistance, S= Susceptible

4.8. Pathogenic potential

The bacterial isolates obtained through streak plating of the respective microcosm incubation period (Day 0, 3, 7, 14, and 28), gathered from the WWTP effluent in Potchefstroom, NWP and the associated river system, had various virulence characterisations. Therefore, the antibiotic-resistance profiles (Table 4.2) and the extracellular enzyme production (Table 4.1) were combined to create a pathogenic potential index (PPI) to differentiate the risk posed by the unidentified isolates (Table 4.3). The Index is a mathematical description of a microbe's ability to cause disease in a host (Smith & Casadevall, 2022).

Isolates exhibiting haemolysis were allocated scores of 0.5 for α- and 1 for β-haemolysis, β-haemolysis was scored higher due to the complete lysis of red blood cells. The positive presence of an extracellular enzyme was allocated a score of 1. The antibiotic-resistance profiles were also scored, if an isolate presented resistance towards an antibiotic, a score of 1 was awarded, 0.5 if the isolate indicated intermediate resistance and a score of 0 was assigned to isolates that were susceptible towards the antibiotics. The isolates that scored 7 or higher underwent further identification.

Table 4.4 Illustrates a summarised version of the 16 potentially pathogenic bacterial isolates that may threaten human health and the possible presence of antibiotic-resistance genes within the aquatic environment. The completed table containing the 51 potentially pathogenic bacteria is provided in Appendix A (Table A2).

The 16 potentially pathogenic bacteria are divided based on the different substrates they were isolated from (Figure 4.11). The comparison of the different substrates indicated that most of the potentially pathogenic bacteria originated from the HDPS and wood biofilm, whilst the remaining substrates had fewer pathogenic bacteria detected.

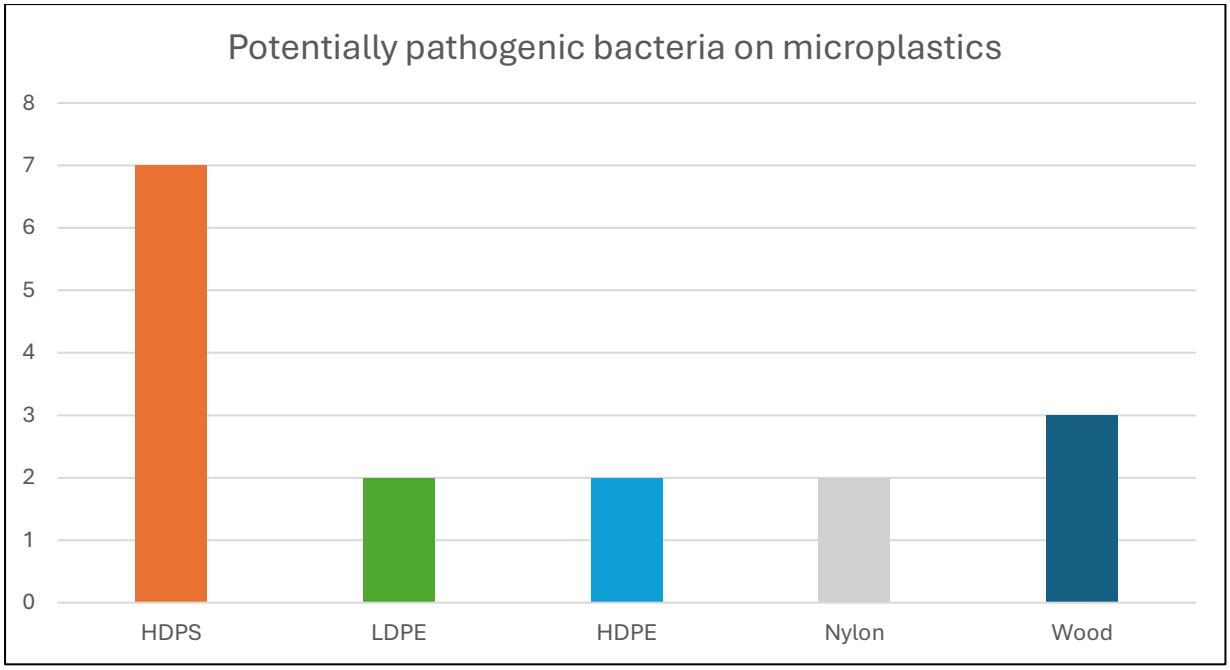


Figure 4.11: Potentially pathogenic bacteria isolated from the different substrates.

Table 4.3: Summarised Pathogenic Potential Index

Isolate number	Virulence Characteristics				Antibiotic Resistance					Pathogen Score
	Haemolysis	Proteinase	DNase	Lipase	VA30	A10	K30	C30	T5	
	β = 1; α = 0.5		X= 1		R=1, I=0.5, S=0					
14	α	X	X	X	R	R	I	S	R	7
16	α	X	X	X	R	R	I	R	R	8
17	α	X	X	X	R	R	S	R	R	7.5
23	α	X	X	X	R	R	R	R	R	8.5
27	α	X	X	X	S	R	R	R	R	7.5
29	α	X	X	X	R	R	R	I	R	8
33	α	X	X	X	R	I	R	S	R	7
36	β	X	X	X	R	R	R	S	R	8
37	β	X	X	X	S	R	I	I	R	7
38	β	X	X	X	R	R	I	S	I	7
39	β	X	X	X	R	R	I	S	I	7
40	β	X	X	X	R	R	I	S	I	7
42	β	X	X	X	R	R	S	R	R	8
44	β	X	X	X	I	R	R	I	R	8
46	α	X	X	X	R	R	R	I	R	8
50	β	X	X	X	S	R	S	R	R	7

R= Resistant, I=Intermediate, S= Susceptible

4.9. Isolate identification

Figure 4.12 is an illustration of the 1.5% agarose gel of the amplicons derived from the PPI. To confirm the identity of the 16 chosen bacterial isolates, the genomic DNA was amplified by PCR and underwent Sanger Sequencing as stated under section 3.6. A 1 Kb and 100 bp ladder was used to indicate the size of the bands (O' GeneRulerTM 1 Kb DNA ladder, Fermentas Life Science, US). All of the illustrated amplicons were in the expected range of 1500 bp with the absence of non-specific bands and primer dimers. As seen in Figure 4.12, no contamination was present due to the absence of a band in the no-template control (C), as seen in the 2nd lane. Visible bands could be seen in the majority of the lanes. Lanes 7, 12, 14, 15, and 16 were amplified unsuccessfully due to the absence of a band. The unsuccessful amplification products were discarded. The unsuccessful amplification led to the re-amplification of these isolates; successful amplification was achieved thereafter.

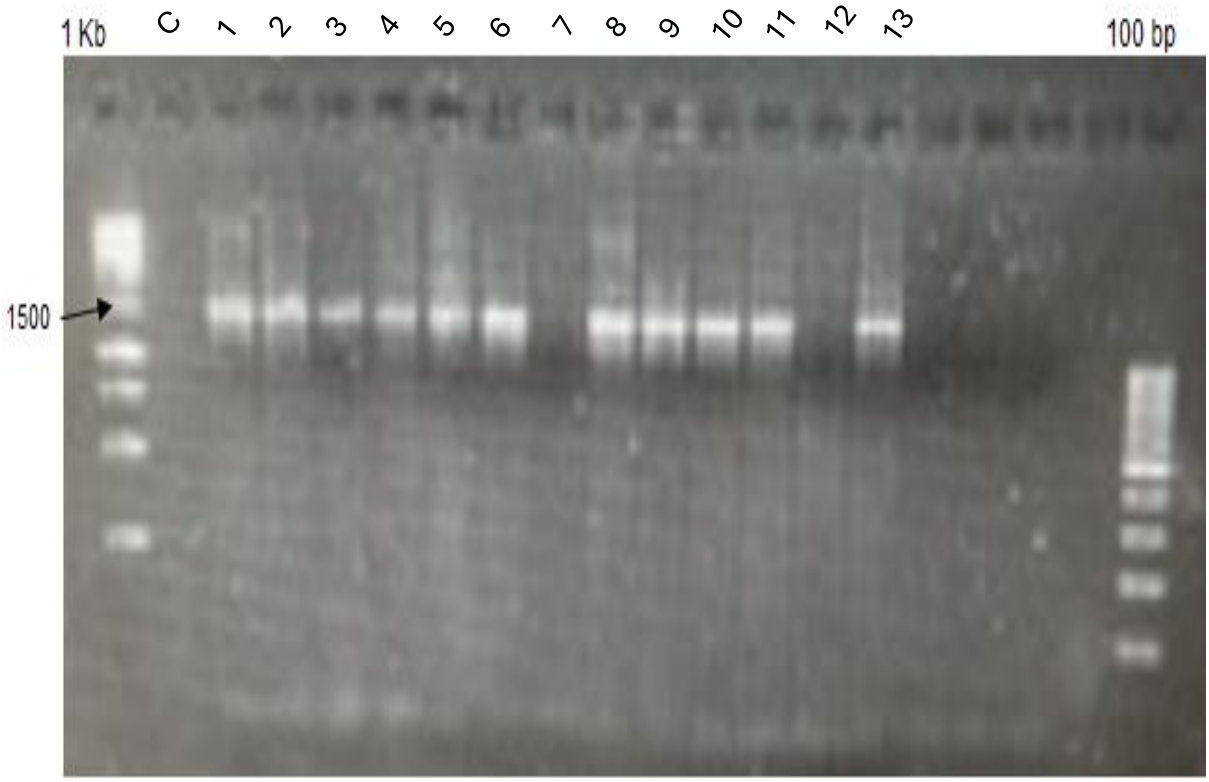


Figure 4.12: 1.5% agarose gel electrophoresis showing examples of the 16S rRNA amplicons used for Sanger sequencing. Lane C represents the No template control, 1 Kb in lane 1 represents the 1 Kb molecular size marker (O' GeneRulerTM 1 Kb DNA ladder, Fermentas Life Science, US). 100 bp represents the 100 bp molecular size marker (Thermo Fisher Scientific 100 bp DNA ladder, Thermo Fischer Scientific, US).

Figure 4.13 illustrates the purified PCR product used for Sanger sequencing. The first PCR cleanup was successful due to the presence of a clear band and the absence of smudging.

The successful PCR products underwent purification and Sanger sequencing. The amplified fragments were sequenced in-house using the SeqStudio Genetic Analyzer (Thermo Fischer Scientific).

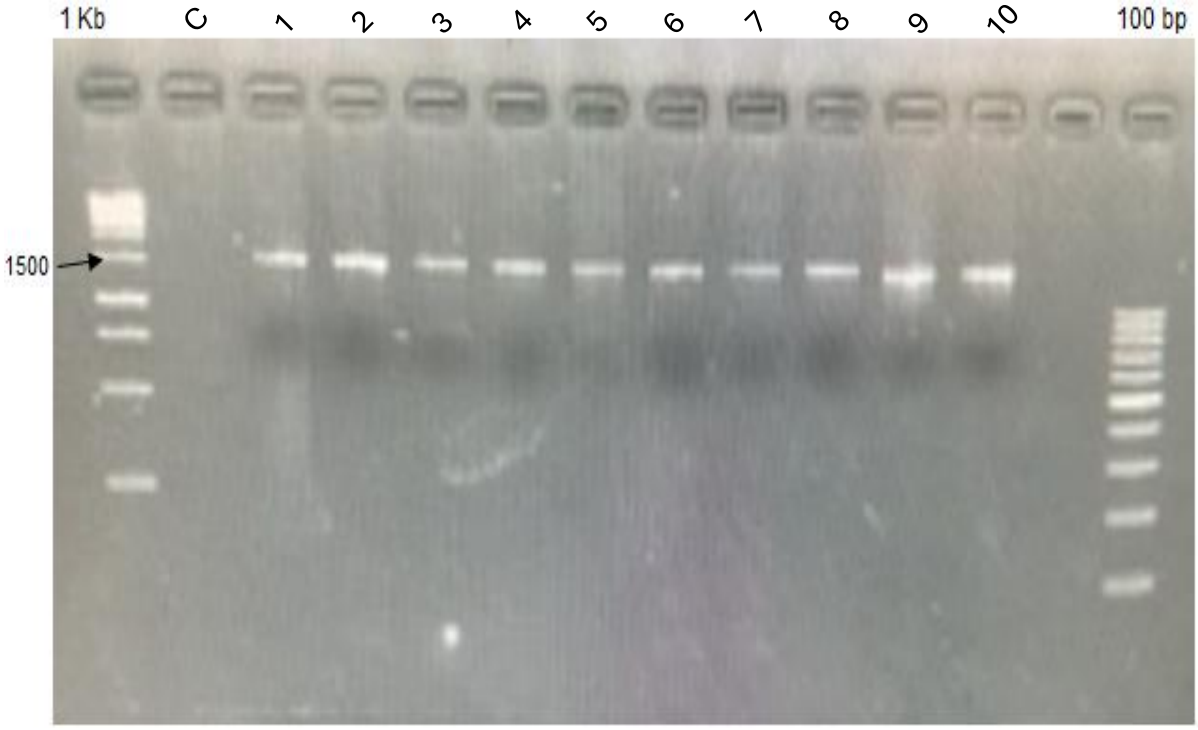


Figure 4.13: Photograph example of the 16rRNA amplicons electrophoresis gel (1.5%) after the First PCR cleanup. Lane C represents the No template control, 1 Kb represents the 1 Kb molecular size marker (O' GeneRulerTM 1 Kb DNA ladder, Fermentas Life Science, US). 100 bp represents the 100 bp molecular size marker (Thermo Fisher Scientific 100 bp DNA ladder, Thermo Fischer Scientific, US).

The chromatograms were viewed using FinchTV and underwent NCBI BLAST searches against the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST), to determine the bacterial isolate identity. The identification of the isolates obtained from various substrates are summarised in Table 4.4. Table A3 in Appendix A include additional information, such as the accession number from the GenBank, the percentage (%) similarity and the type of substrate associated with the isolate.

The identification of the isolated bacterial samples is indicated in Table 4.4. The successful sanger sequences were imported from the NCBI to Mega BLAST; thereafter, identification was made possible. Five different genera were identified. The bacterial isolates identified include *Aeromonas hydrophilia*, *Aeromonas caviae*, *Aeromonas veronii*, *Providencia vermicola*, *Providencia rettgeri*, *Stenotrophomonas koreensis*, *Brevundimonas diminuta*, and *Bacillus subtilis*.

Table 4.4: Bacterial isolate identification.

ISOLATE NUMBER	IDENTIFICATION	SUBSTRATE
14	*Aeromonas hydrophila* strain ATCC 7966	HDPS
16	*Aeromonas hydrophila* strain ATCC 7966	LDPE
17	*Aeromonas caviae* strain ATCC 15468	LDPE
23	*Stenotrophomonas koreensis* strain TR6-01	Nylon
27	*Aeromonas caviae* strain CECT 4221	HDPS
29	*Bacillus subtilis* strain IAM 12118	W
33	*Stenotrophomonas koreensis* strain TR6-01	Nylon
36	*Aeromonas hydrophila* strain ATCC 7966	HDPS
37	*Brevundimonas diminuta* strain NBRC 12697	HDPS
38	*Aeromonas hydrophila* strain ATCC 7966	HDPS
39	*Aeromonas hydrophila* strain ATCC 7966	HDPS
40	*Aeromonas hydrophila* strain ATCC 7966	HDPS
42	*Providencia vermicola* strain OP1	HDPE
44	*Aeromonas veronii* strain JCM 7375	HDPE
46	*Providencia rettgeri* strain DSM 4542	W
50	*Brevundimonas diminuta* strain NBRC 12697	W

4.10. Phylogenetic analysis

The sequences were compared to 16S rRNA gene sequences within the GenBank database through BLAST searches. The sequences with high similarities (≥ 94%) were used for phylogenetic analysis. The sequencing results were used to construct the phylogenetic tree are found in Table A3 in Appendix A. MEGA 11 was used to identify and predict a phylogenetic tree for the relatedness of the isolates by testing the maximum likelihood of the sequences and bootstrapping of 1000 replicates were performed (Tamura *et al*., 2021) (Figure 4.14).

Figure 4.14 illustrates the phylogenetic relationship of the nine isolated bacteria, excluding the duplicates. The species from the same genus were tested against Tamura – Nei model and a maximum likelihood phylogenetic tree was constructed (Tamura & Nei, 1993). This is

supported by the strong bootstrap values, with the majority of the species grouping between 96% and 100%.

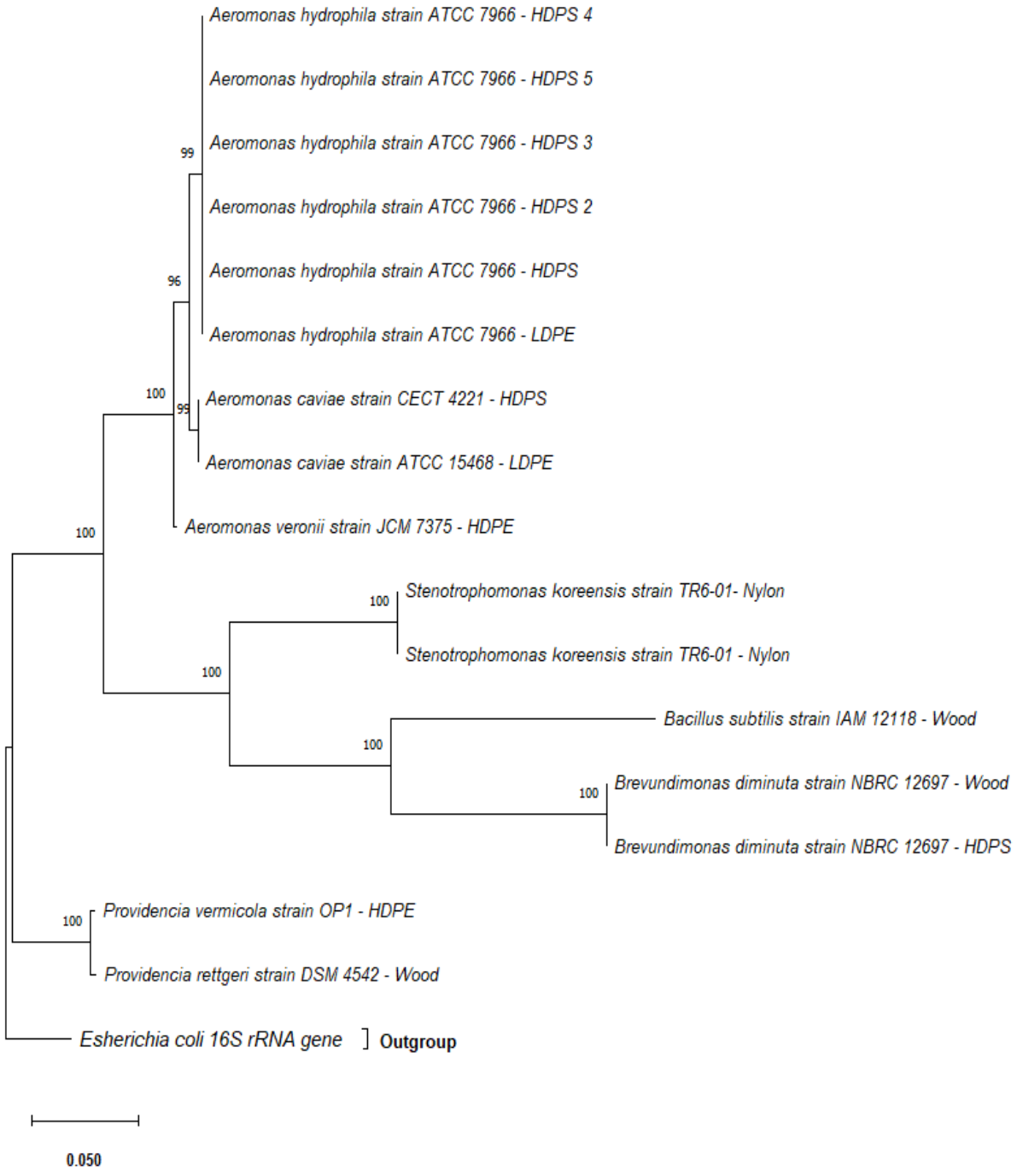


Figure 4.14: Maximum likelihood Phylogenetic tree representing the relationship of 16S gene sequences from the GenBank database and the sequences of pathogenic bacteria isolated from the simulated freshwater environment. *Escherichia coli* was used as an outgroup.

The scale in the tree is 0.05, thereby indicating that there is less than 1% genetic change which indicates minimum evolutionary change and a common ancestor between the various genera found. The bootstrap percentage is indicated on the branches (Figure 4.14).

In Figure 4.14, some of the relationships observed are briefly discussed below. *Bacillus subtilis* was seen to be clustered with *Brevundimonas diminuta* with 100% bootstrap support. This indicates a common ancestor between the species. *Stenotrophomonas koreensis* is seen to diverge from the *Bacillus* spp. and *Brevundimonas diminuta* cluster, which is supported by the strong bootstrap value (100%). *Providencia rettgeri* and *Providencia vermicola* are seen to be clustered together with 100% bootstrap support. *Aeromonas caviae* strain CECT 4221 and *Aeromonas caviae* strain 15468 are clustered together with a high bootstrap support of 99%, indicating the evolutionary relatedness between these bacteria. *Aeromonas veronii* strain JCM 7375 is seen to form a separate cluster with 100% bootstrap support. clade with *Aeromonas hydrophila* strain ATCC 7966 and *Aeromonas caviae* with a strong bootstrap support of 100%. All four strains are grouped together with a bootstrap value of 100%.

Table 4.5 provides a summary of the identified potential pathogens for this study, and the potential health effects associated with these pathogens, as well as the virulence of these bacteria. It can be observed that *Stenotrophomonas koreensis* strain TR6-01 had the highest potential for being pathogenic as compared to the other species. *Aeromonas hydrophila* strain ATCC 7966, *Bacillus subtilis* strain IAM 12118, *Providencia vermicola* strain OP, *Aeromonas veronii* strain JCM 7375 and *Providencia rettgeri* strain DSM 4542 also illustrated potential pathogenicity.

Table 4.5: Summary of characteristics of the potentially pathogenic bacteria

Identification	Gram stain	Extracellular enzymes	Antibiotic resistance	Possible health effects	Reference
Aeromonas hydrophila strain ATCC 7966 (6)	-	DN, He, Li, Pr	VA30, A10, K30, T5	Gastrointestinal issues, meningitis, septicaemia, cholera-like illness	(Grim *et al*., 2013; Nhinh *et al*., 2021)
Aeromonas caviae strain ATCC 15468	-	DN, He, Li, Pr	VA30, A10, C30, T5	Gastrointestinal issues, Acute diarrhoea	(Monfort & Baleux, 1991; Namdari & Bottone, 1990; Wang *et al*., 1996; Vila *et al.,* 2002)
Stenotrophomonas koreensis strain TR6-01 (2)	-	DN, He, Li, Pr	VA30, A10, C30, K30, T5	Respiratory tract infections, nosocomial infection	(Wang *et al*., 2017)
Aeromonas caviae strain CECT 4221	-	DN, He, Li, Pr	A10, K30, C30, T5	Gastrointestinal issues, Acute diarrhoea	(Monfort & Baleux, 1991; Namdari & Bottone, 1990; Wang *et al*., 1996; Vila *et al*., 2002)
Bacillus subtilis strain IAM 12118	+	DN, He, Li, Pr	VA30, A10, K30, C30, T5	Pneumonia, bacteraemia, sepsis, meningitis	(Al-Habibi *et al*., 2022)
Brevundimonas diminuta strain NBRC 12697 (2)	-	DN, He, Li, Pr	A10, K30, C30, T5	Nosocomial infection, Peritonitis	(Ryan & Pembroke, 2018)
Providencia vermicola strain OP1	-	DN, He, Li, Pr	VA30, A10, C30, T5	Urinary Tract infections, nosocomial infections	(Lupande-Mwenebitu *et al*., 2021; Somvanshi *et al*., 2006; Sharma *et al*., 2017)
Aeromonas veronii strain JCM 7375	-	DN, He, Li, Pr	VA30, A10, K30, C30, T5	Gastrointestinal issues	(Monfort & Baleux, 1991; Namdari & Bottone, 1990; Wang *et al*., 1996; Vila *et al*., 2002)
Providencia rettgeri strain DSM 4542	-	DN, He, Li, Pr	VA30, A10, K30, C30, T5	Gastrointestinal tract infections, nosocomial infections	(Trivedi *et al*., 2015; Sharma *et al*., 2017)

A10: Amoxicillin, C30: Chloramphenicol, DN: DNase, +: Gram Positive, -: Gram Negative, Ha: Haemolysis, K30: Kanamycin, Li: Lipase, Pr: Proteinase, VA30: Vancomycin, T5: Trimethoprim. The number in brackets represents the number of isolates that were identified for that organism.

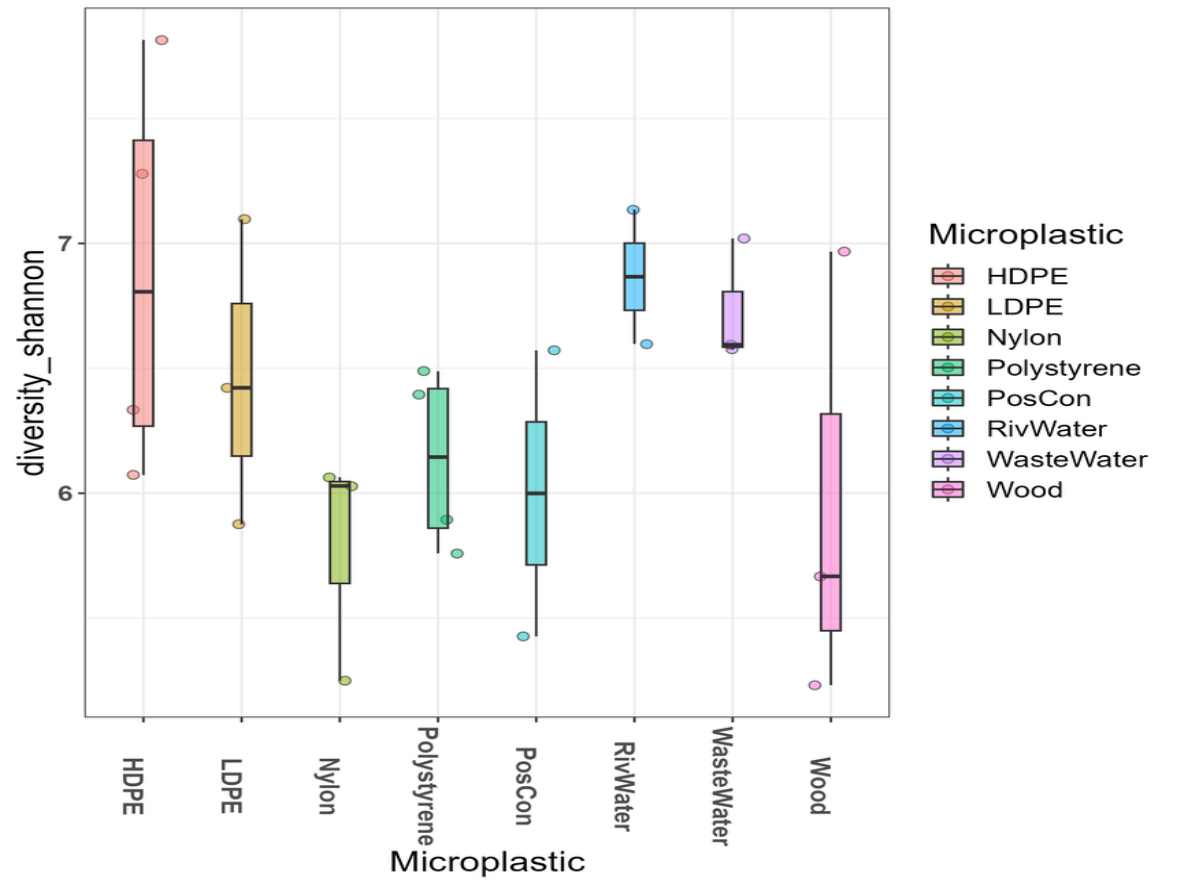
4.11. Biofilm composition characterisation

DNA extraction of the biofilms surrounding the various substrates was conducted and stored at -4°C. Next-generation sequencing was performed in-house through the use of the Illumina Miseq machine (Illumina, USA). The bacterial community composition was investigated, and the diversity was determined.

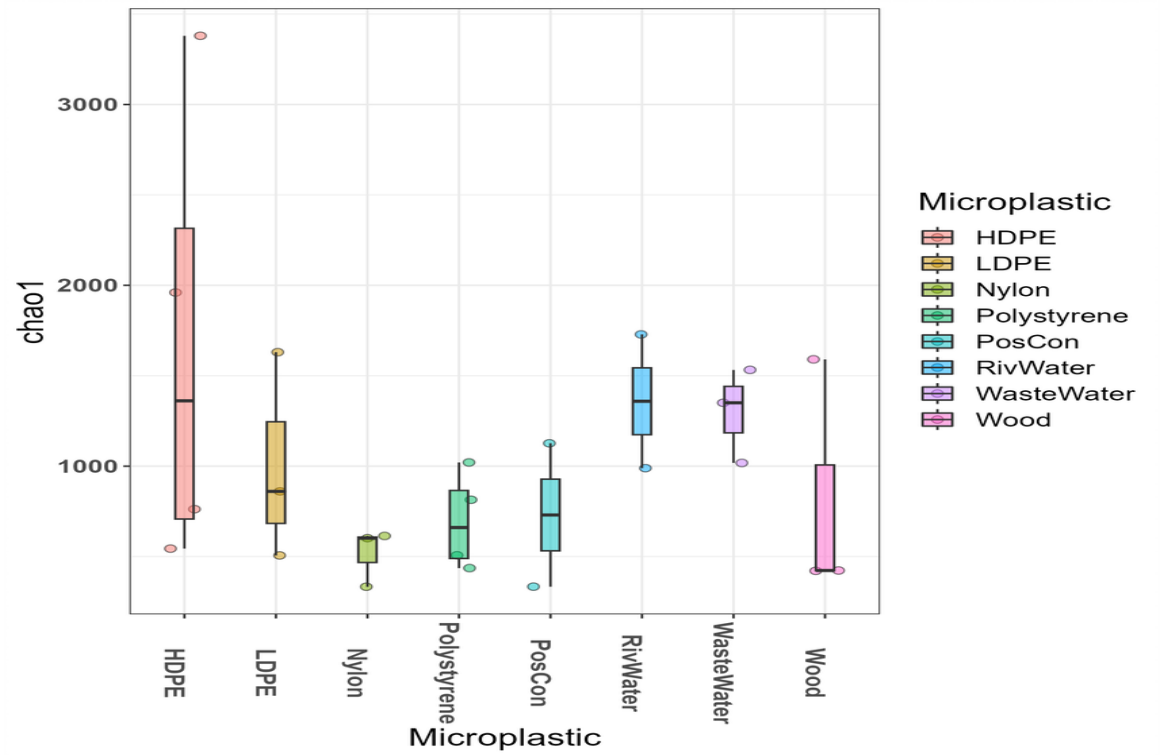
4.11.1. Bacterial community comparison

Alpha diversity and significance tests were calculated for species richness (Chao1 estimator), species diversity and evenness (Shannon Index), and species dominance (Simpson Index) for sample type (microplastics, wood, and filtrate) (Figure 4.15) and in doing so, determining the environmental conditions. Figure 4.16 illustrates the Beta Diversity of the isolated samples through the Jaccard similarity matrices.

a)



b)



c)

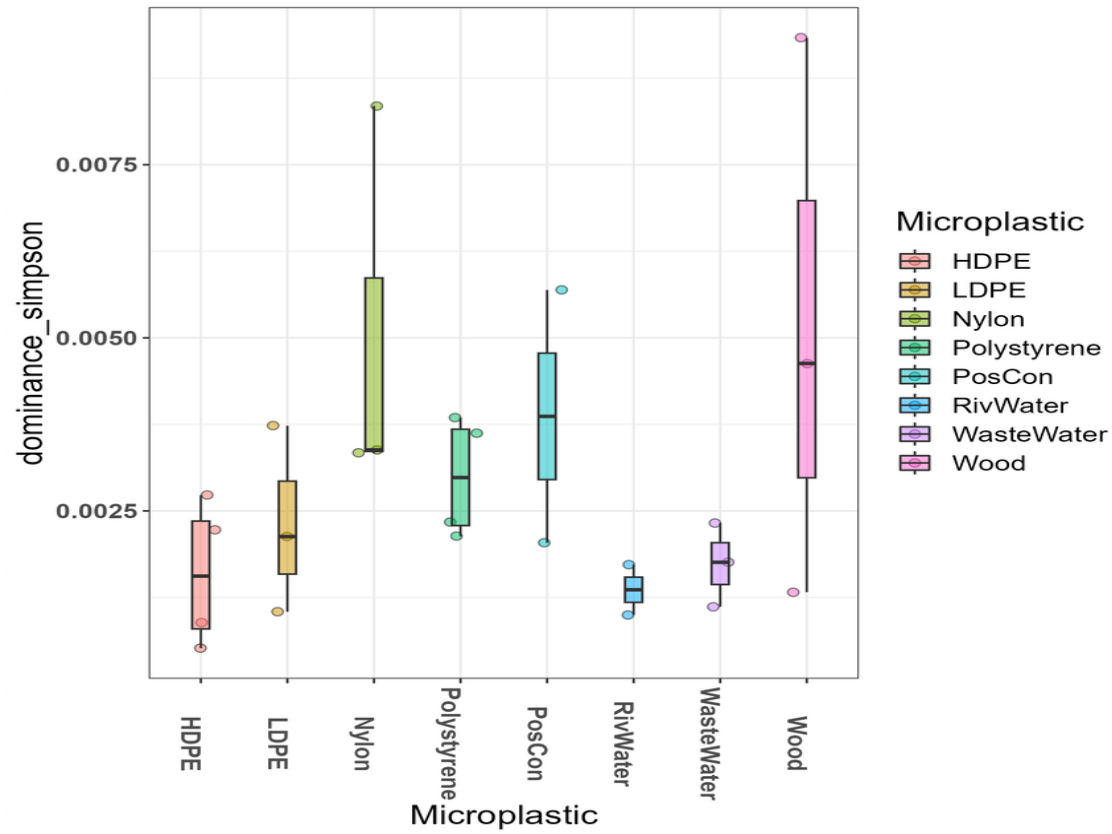


Figure 4.15: Alpha diversity indices (Shannon: a, Chao1: b and Simpson: c) for the sample type (microplastics, wood and water sources) Poscon – positive control; RivWater – river water.

Figure 4.15 a) indicates that the Shannon Index value varied between the different substrates and water sources. The Shannon – Weaver Diversity Index indicated a diversity range between 5 and 8, with the most diversity observed on the river water, while the Positive control sample had the least diversity detected.

Figure 4.15 b) illustrates the estimated species richness (Chao1 Estimator) on the different substrates. River water and Wastewater presented the richest bacterial community, whilst Nylon had the lowest species richness.

Figure 4.15 c) indicates the Simpson Dominance Index, the dominance of the species found within the bacterial community. Wood indicated the higher species dominance compared to the River Water sample, which had lower species dominance. Based on the species richness, diversity and dominance, there is diversity in microbial community observed between the substrates.

Considering all the substrate types, the results demonstrated that the river water samples had the highest species diversity and richness, and wood presented the highest species dominance. The positive control presented a higher species dominance, but a decreased species diversity and richness compared to the WWTP effluent sample.

Figure 4.16 indicates the Beta Diversity of the isolated samples through the Jaccard similarity index. The Jaccard similarity index was used to demonstrate the community composition between the various substrates. The majority of the substrates indicated a similar community composition except for the wood and wastewater samples. The nMDS plot indicated an overlap between the HDPS, HDPE, nylon and LDPE bacterial communities while the positive control, river water and wood samples formed more discrete groups in the ordination space and can be attributed to the limited community overlap. The community composition between the different wood samples indicated variation in community composition.

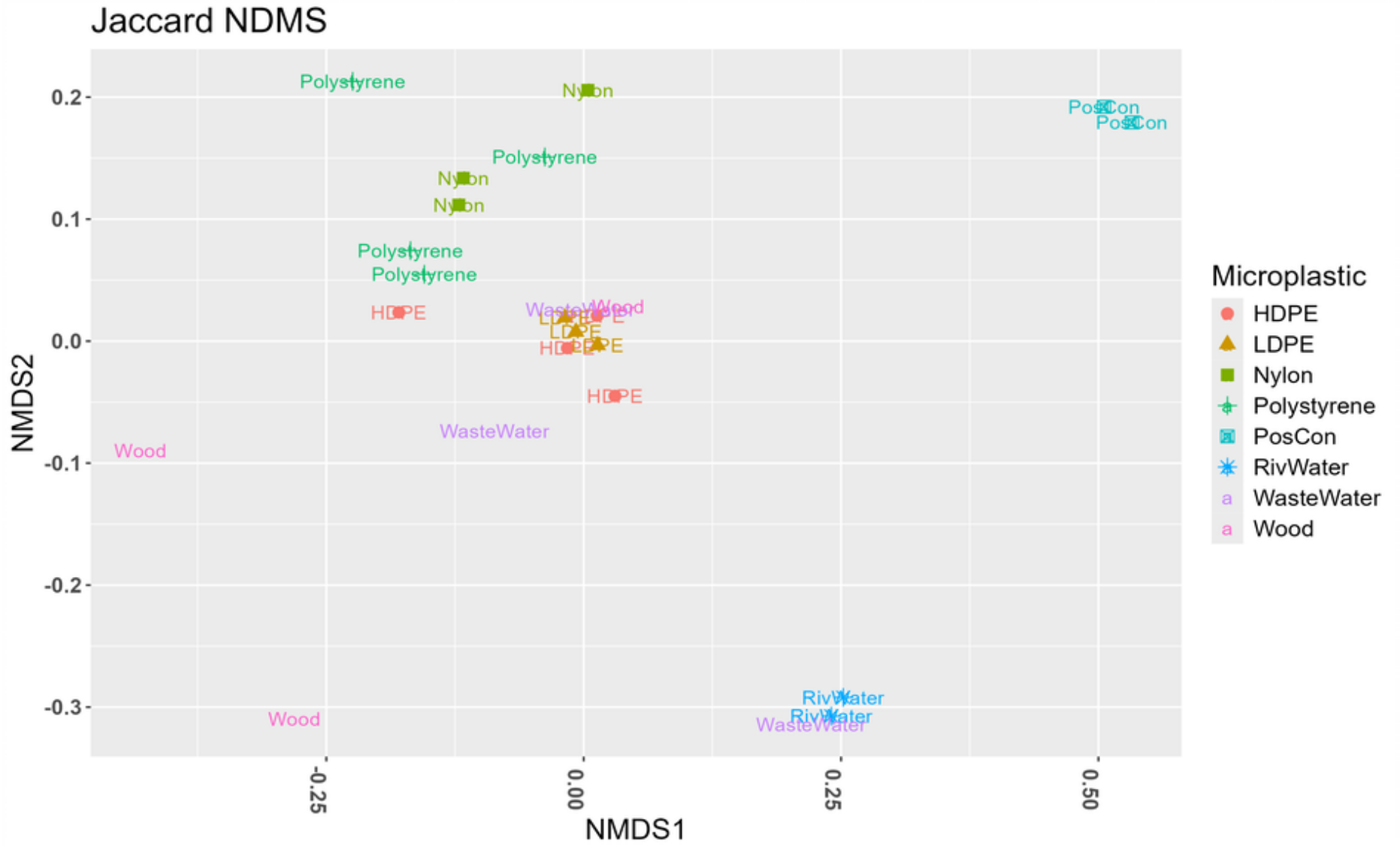


Figure 4.16: Statistical representation of the occurrence of bacterial data obtained from next-generation sequencing.

4.11.2. Bacterial community membership

The bacterial community membership across the various substrates was identified according to the kingdom, phylum, class, order, family, genus and species. The classification presented an Unassigned portion which can be attributed to the limited isolates identified. The kingdom abundance of the microbes detected in the biofilms surrounding the different substrates indicated that Bacteria the prevalent kingdom is.

Figure 4.17 indicates the taxonomy of detected microbes based on their phylum. Based on the data obtained from NGS, 14 different phyla were detected across the different water sources and substrates. The different phyla include Acidobacteriota, Actinobacteriota, Bacteroidota, Campylobacterota, Chloroflexi, Cyanobacteria, Desulfobacterota, Firmicutes, Gemmatimonadota, Nitrospirota, Patescibacteria, Planctomycetota, Proteobacteria, and Verrucomicrobiota.

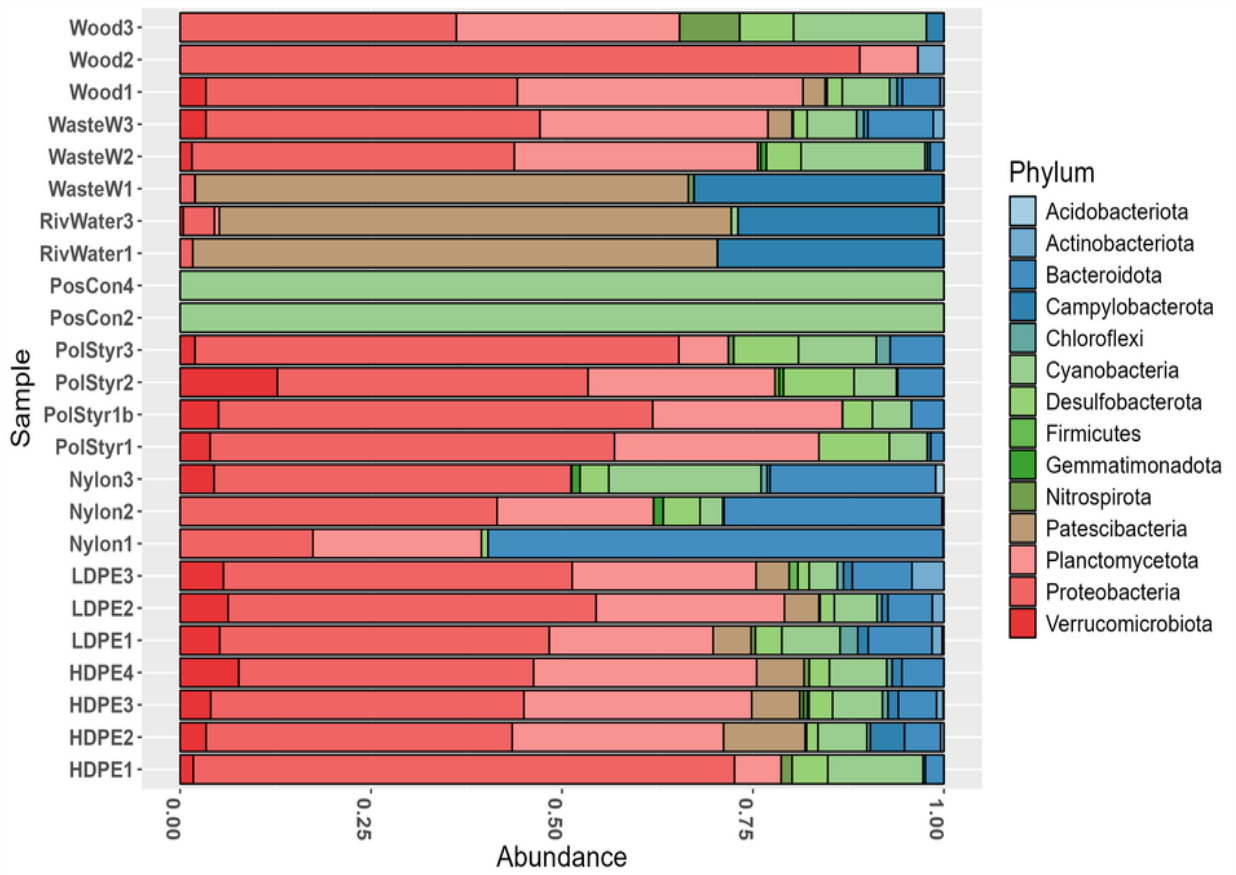


Figure 4.17: Relative abundance of microbial colonisation as different phyla.

The Bacterial community composition varied among the different substrates and water sources (Figure 4.17). Positive control samples 2 and -4 consist of 100% of Cyanobacteria. The majority of the different substrates illustrated that Proteobacteria was the most dominant

phyla (37.77%), followed by Planctomycetota (17.69%) on the various microplastics. Bacteroidota was the most dominant phylum found in the nylon 1 biofilm compared to Patescibacteria in the river water and WWTP effluent sources, whilst the least abundant phyla (≤1%) included, Nitrospirota, Actinobacteriota, Chloroflexi, Gemmatimonadota, Acidobacteriota and Firmicutes from the various substrates. Wood substrates recovered from the microcosm were dominated by the following phyla: Proteobacteria (55.30%), Planctomycetota (24.73%), Cyanobacteria (8.70%), Nitrospirota (3.93%), Desulfobacterota (3.52%), Actinobacteriota (1.71%) and Campylobacterota (1.13%).

In the river water samples, the dominant phyla detected included Patescibacteria (67.86%), Campylobacterota (27.94%), and Proteobacteria (2.87%). The wastewaters 2 and -3 samples were dominated by Proteobacteria (42.97%), Planctomycetota (30.84%), and Cyanobacteria (11.35%). The wastewater 1 sample was dominated by Patescibacteria (64.57%), Campylobacterota (32.56%), and Proteobacteria (1.92%) (Figure 4.17).

The HDPE polymer was dominated by the following phyla: Proteobacteria (47.64%), Planctomycetota (23.20%), and Cyanobacteria (8.23%). The LDPE substrates were dominated by Proteobacteria (45.67%), Planctomycetota (23.40%), and Bacteroidota (7.33%). The nylon 2 and -3 substrates were dominated by the following phyla: Proteobacteria (44.12%), Bacteroidota (25.11%), Cyanobacteria (11.45%), and Planctomycetota (10.27%). The nylon 1 substrate was dominated by the following phyla, Bacteroidota (59.58%), Planctomycetota (22.05%), and Proteobacteria (17.39%). The HDPS polymer was dominated by Proteobacteria (53.45%), Planctomycetota (20.64%), and Desulfobacterota (7.71%) (Figure 4.17). The results indicated that the microbial composition amongst the various substrates varied.

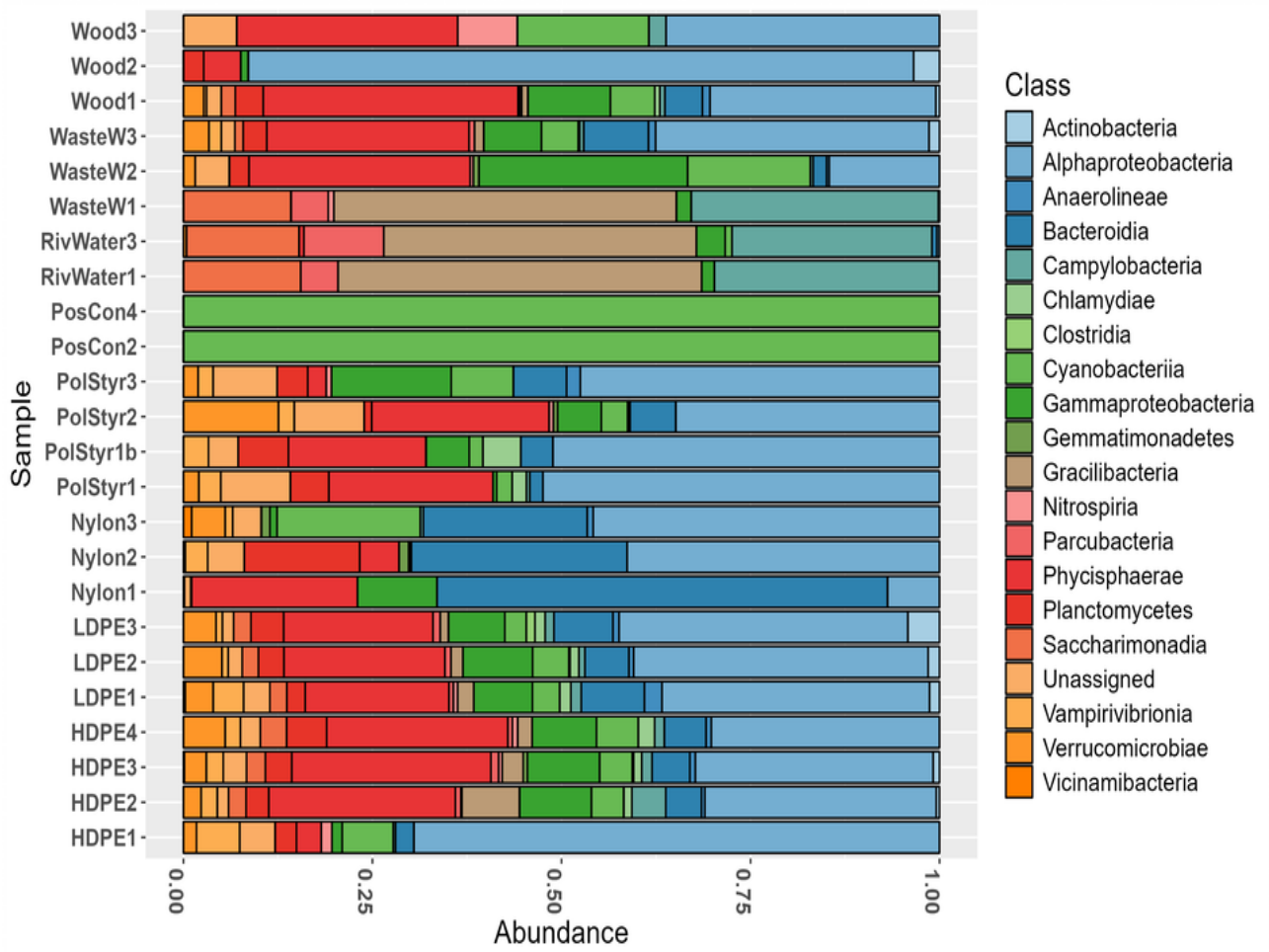


Figure 4.18: Relative abundance class based on taxonomic classification.

Figure 4.18 indicates the taxonomy of detected microbes based on their class. The different classes present in the microbial community include Actinobacteria, Alphaproteobacteria, Anaerolineae, Bacteroidia, Campylobacteria, Chlamydiae, Clostridia, Cyanobacteriia, Gammaproteobacteria, Gemmatimonadetes Gracilibacteria, Nitrospiria, Parcubacteria, Phycisphaerae, Planctomycetes, Saccharimandia, Vampirivibrionia, Verrucomicrobiae, Vicinamibacteria along with small a portion classified as unassigned. Positive control 2 and 4 bacterial class composition consisted of Cyanobacteriia (100%).

The HDPE polymer was dominated by the following classes: Alphaproteobacteria (40.44%), Phycisphaerae (19.57%), and Gammaproteobacteria (7.22%). The LDPE substrate was dominated by Alphaproteobacteria (37.51%), Phycisphaerae (20.02%), Gammaproteobacteria (8.15%), and Bacteroidia (7.33%). The dominant class distribution of the microbes on the nylon 1 polymer is as follows: Bacteroidia (59.58%), Phycisphaerae (21.94%), Gammaproteobacteria (10.51%), and Alphaproteobacteria (6.87%). Alphaproteobacteria (43.56%), Bacteroidia (25.11%), Cyanobacteriia (9.48%), and Planctomycetes (7.63%) were the dominant classes detected of the microbes on the nylon 2

and -3 sample The HDPS polymer's most dominant classes are as follows: Alphaproteobacteria (46.49%), Phycisphaerae (16.45%), Unassigned (7.71%), and Gammaproteobacteria (6.96%) (Figure 4.18).

The microbial community of the river water filtrate samples were dominated by the following classes: Gracilibacteria (44.73%), Campylobacteria (28.09%), Saccharimonadia (15.20%), and Parcubacteria (7.74%). The wastewater 2 and -3 filtrate samples were dominated by the Phycisphaerae (28.01%), Alphaproteobacteria (25.39%), Gammaproteobacteria (17.62%), and Cyanobacteriia (10.55%). The wastewater 1 filtrate sample differs from the wastewater 2 and -3 filtrate sample, the most dominant classes are Gracilibacteria (45.31%), Campylobacteria (32.70%), and Saccharimonadia (14.21%). The wood substrate recovered from the respective microcosm consisted of Alphaproteobacteria (51.35%), Phycisphaerae (22.61%), and Cyanobacteria (7.75%) (Figure 4.18).

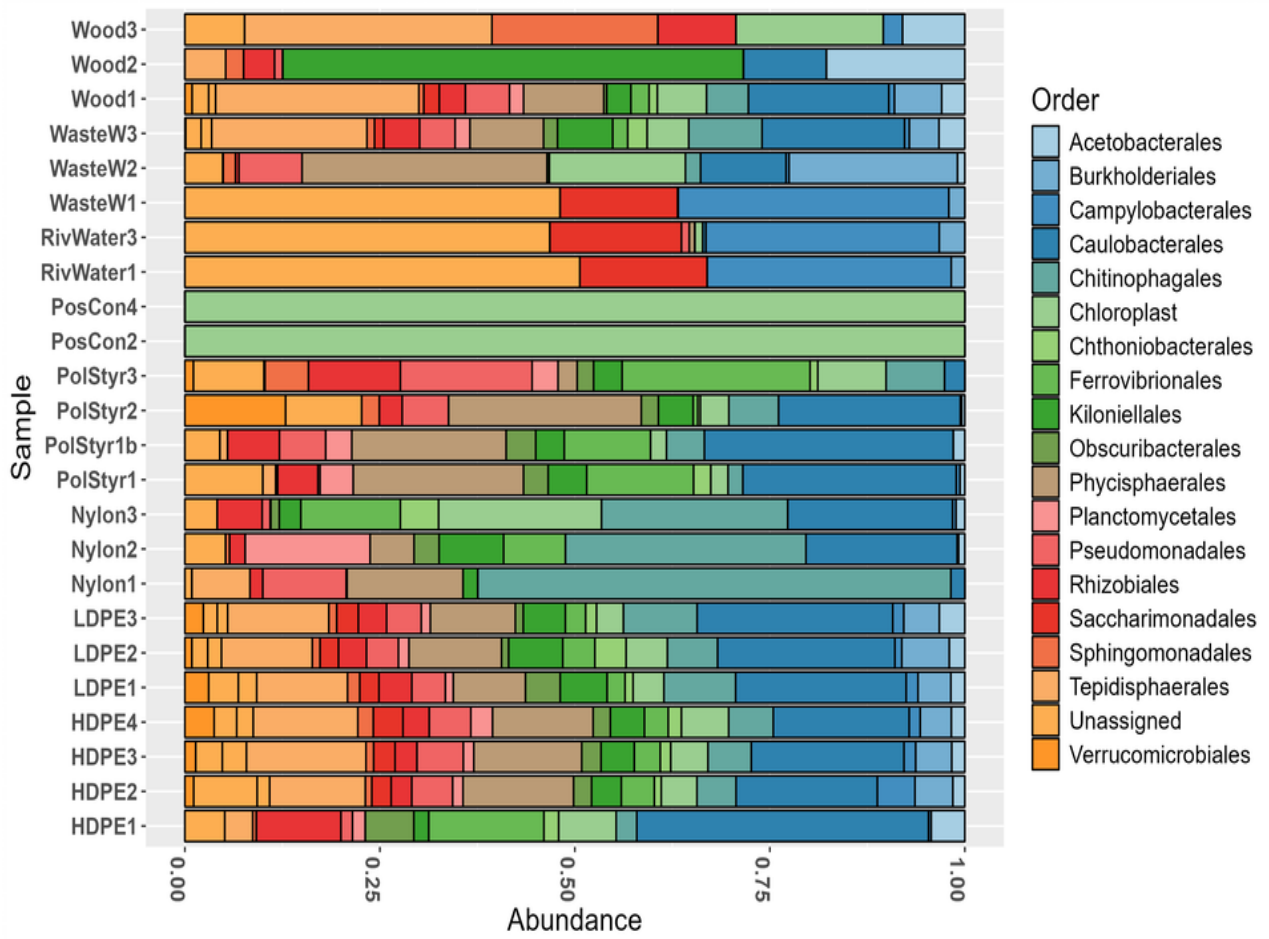


Figure 4.19: Relative abundance order based on taxonomic classification.

Figure 4.19 indicates the taxonomy of the detected microbes based on their order. The different orders found include Acetobacterales, Burkholderiales, Campylobacterales, Caulobacterales, Chitinophagales, Chloroplast, Chthoniobacterales, Ferrovibrionales,

Kiloniellales, Obscuribacterales, Phycisphaerales, Planctomycetales, Pseudomonadales, Rhizobiales, Saccharimonadales, Sphingomonadales, Tepidispaerales, and Verrucomicrobiales along with a small portion classified as unassigned. The relative abundance of the orders found within the biofilms surrounding Positive Control 2 and 4 consisted of Chloroplast (100%). Caulobacterales (14.50%) and Chloroplast (13.42%) were the most dominant orders at the various substrates and water sources.

The dominant orders identified within the microbial community surrounding the HDPE polymer include: Caulobacterales (23.13%), Tepidisphaerales (11.15%), Phycisphaerales (10.23%), Ferrovibrionales (6.35%), and Chloroplast (5.69%). Unassigned orders were detected (6.58%) within the bacterial community surrounding the HDPE substrate. The LDPE polymer was dominated by the following orders: Caulobacterales (23.23%), Tepidisphaerales (12.09%), Phycisphaerales (10.68%), Chitinophagales (8.36%), and Kiloniellales (6.12%). An unassigned portion was detected within the microbial community surrounding the LDPE substrate. Caulobacterales (21.29%), Phycisphaerales (17.20%), Ferrovibrionales (12.37%), Pseudomonadales (7.25%), Rhizobiales (6.63%), Chitinophagales (5.15%), Chloroplast and Kiloniellales (4.17%), is seen as the dominant orders found on the HDPS polymer. A portion of the microbial community could not be identified at order level (8.31%). The microbial community surrounding the nylon polymer was dominated by the following orders: Chitinophagales (38.46%), Caulobacterales (14.10%), Chloroplast (6.96%), Ferrovibrionales (6.89%), Phycisphaerales (6.86%), Planctomycetales (5.38%), and Kiloniellales (4.31%). Unclassified orders were also detected (3.41%) (Figure 4.19).

Tepidisphaerales (21.00%), Kiloniellales (20.73%), Acetobacterales (9.57%), Caulobacterales (9.54%), Chloroplast (8.40%), and Sphingomonadales (8.06%) were the most dominant bacterial orders identified from the bacterial DNA isolated from the wood substrates. Environmental DNA from the river water filtrate illustrated that the top three orders identified were Campylobacterales (30.61%), Saccharimonadales (16.59%) and Burkholderiales (2.51%). A large portion of the bacterial orders detected could not be classified (48.71%). Wastewater filtrate samples were dominated by the following orders: Phycisphaerales (13.63%), Campylobacterales (11.89%), Caulobacterales (9.74%), Burkholderiales (9.15%), Chloroplast (7.59%), Tepidisphaerales (6.65%), Saccharimonadales (5.43%), and Pseudomonadales (4.21%). Unassigned orders were also detected and made up 9.38% of the total microbial composition (Figure 4.19).

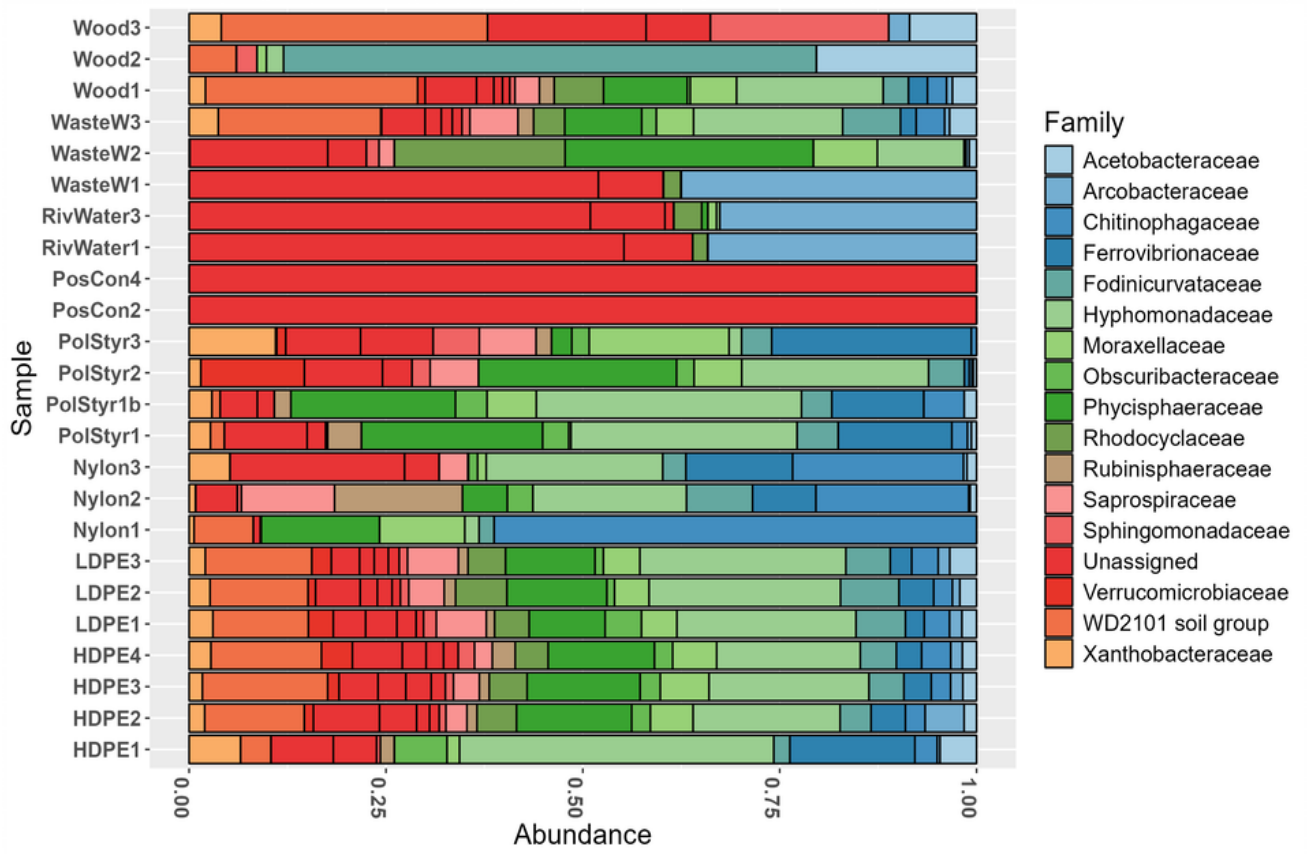


Figure 4.20: Relative abundance family based on taxonomic classification.

Figure 4.20 indicates the taxonomy of the detected microbes based on the family level. Acetobacteraceae, Arcobacteraceae, Chitinophagaceae, Ferrovibrionaceae, Fondinicurvataceae, Hyphomonadaceae, Moraxellaceae, Obscuribacteraceae, Phycisphaeraceae, Rhodocyclaceae, Rubinisphaeraceae, Saprospiraceae, Sphingomonadaceae, Verrucomicrobiaceae, WD2101 soil group, Xanthobacteraceae were detected as well as Unassigned taxonomic families. Hyphomonadaceae (14.71%) is the most abundant family after the Unassigned taxonomic families. It should be noted that the high abundance of genera consisting of unclassified microbes is due to the complexity and uniqueness of the microbiome surrounding the substrates and the incomplete sequencing of the plastisphere, resulting in unsuccessful classification (Nasir *et al*., 2023). Positive controls 2 and 4 consisted fully of the unassigned family.

The microbial community surrounding the HDPE polymer were classified on the family level, the most dominant families identified include Hyphomonadaceae (24.29%), WD2101 soil group (11.61%), Phycisphaeraceae (10.62%), and Ferrovibrionaceae (6.72%). Unclassified families were also detected (14.08%). The most dominant families identified from the LDPE polymer are as follows: Hyphomonadaceae (24.41%), WD2101 soil group (12.70%), Phycisphaeraceae (11.23%), Fodinicurvataceae (6.44%), Saprospiraceae (5.77%), and

Rhodocyclaceae (5.22%). A portion of unassigned families were detected within the biofilm surrounding the LDPE substrates (10.29%). Hyphomonadaceae (21.92%), Phycisphaeraceae (17.91%), Ferrovibrionaceae (13.02%), and Moraxellaceae (7.58%) were the dominant families identified within the HDPS biofilm. The unidentified families comprised 6.51% of the total plastisphere surrounding the HDPS polymer. The most dominant families identified from the nylon biofilms are Chitinophagaceae (34.13%), Hyphomonadaceae (14.58%), Ferrovibrionaceae (7.19%), Phycisphaeraceae (6.93%), Rubinisphaeraceae (5.45%), and Saprospiraceae (5.15%). Unidentified families were also detected (10.91%) (Figure 4.20).

The non-plastic control substrate is dominated by the following bacterial families: Fodinicurvataceae (23.62%), WD2101 soil group (22.25%), Acetobacteraceae (10.64%), Sphingomonadaceae (8.64%), and Hyphomonadaceae (6.93%). A portion of unidentified families were also detected (13.01%). The river water filtrate sample consisted of bacterial unidentified families (62.74%), followed by Arcobacteraceae (33.36%) and Rhodocyclaceae (2.73%). The WWTP effluent filtrate sample consisted of the following families: Phycisphaeraceae (13.79%), Arcobacteraceae (12.83%), Hyphomonadaceae (9.98%), Rhodocyclaceae (9.28%), and WD2101 soil group (6.89%) (Figure 4.20). The microbial community profiling revealed that 30.95% consisted of unclassified families.

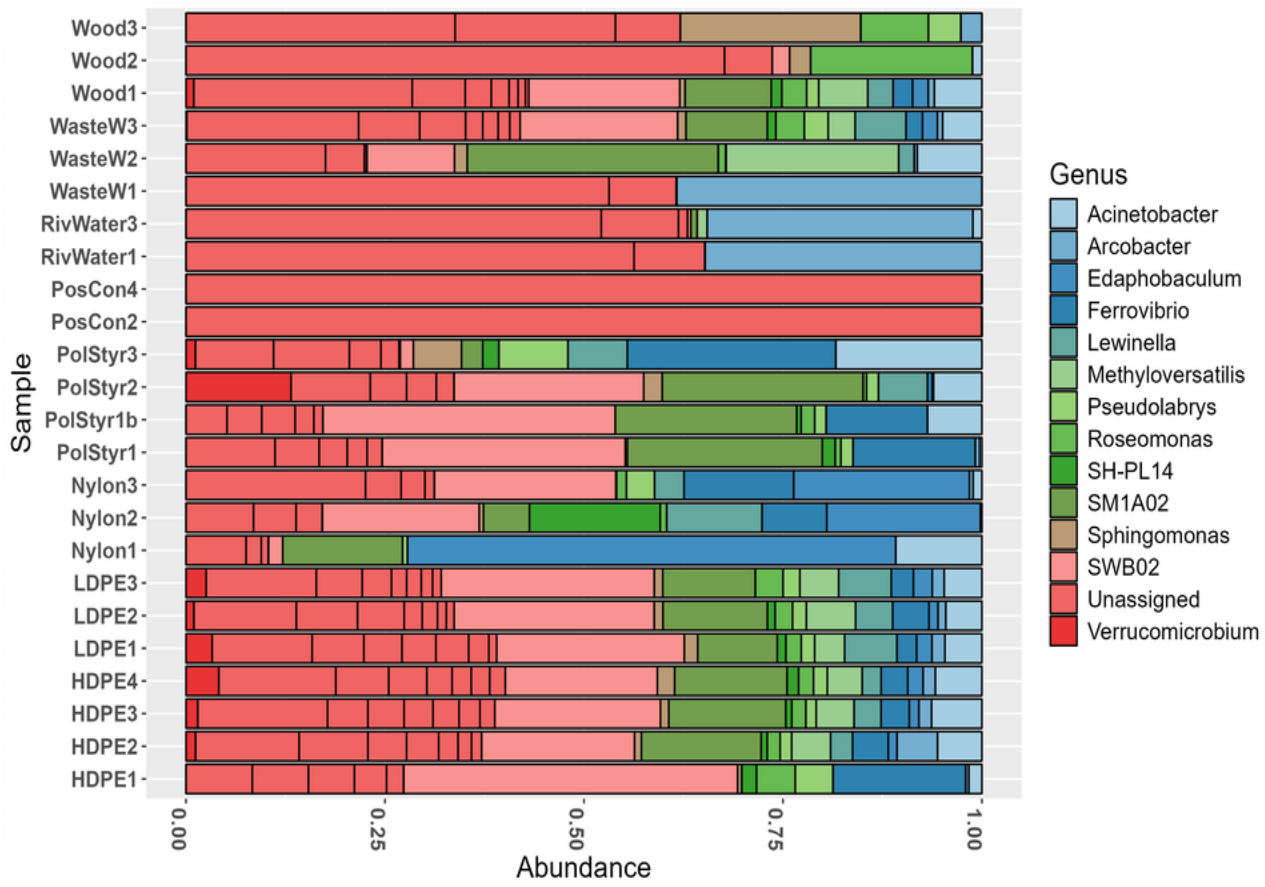


Figure 4.21: Relative abundance of microbial colonisation as different genera.

Figure 4.21 presents the different genera present in the biofilm surrounding the different substrates and water sources. From the isolated bacterial samples, 14 different genera were detected from the microbial communities surrounding the microplastics. *Acinetobacter*, *Acrobacter*, *Edaphobaculum*, *Ferrovibrio*, *Lewinella*, *Methyloversatilis*, *Pseudolabrys*, *Roseomonas*, *SH-PL14*, *SM1A02*, *Sphingomonas, SWB02, Verrucomicrobium* and unassigned genus. The most abundant genera are *SWB02, SM1A02, Ferrovibrio* and *Edaphobaculum,* along with unassigned portions. Unassigned genera were detected at positive controls 2 and 4

The most dominant genera detected within the HDPE plastisphere are as follows: *SWB02* (25.27%), *SM1A02* (10.97%), *Ferrovibrio* (7.02%) and *Acinetobacter* (4.85%) whilst the unclassified genera consisted of 34.14% of the microbial community. The microbial community surrounding the LDPE polymer can be divided into the following genera in order of most dominant, unassigned genera (32.66%), *SWB02* (25.17%), *SM1A02* (11.58%), *Lewinella* (5.95%), *Methyloversatilis* (4.94%), and *Acinetobacter* (4.64%). The most dominant bacterial genera within the nylon plastisphere detected are as follows: *Edaphobaculum* (34.24%), *SWB02* (14.78%), *Ferrovibrio* (7.30%), *SM1A02* (6.96%), *SH-PL14* (5.47%), and *Lewinella* (5.22%). Unclassified bacterial genera were also detected within the biofilm surrounding the nylon polymer and made up 19.55% of the total microbial community. The HDPS biofilm was dominated by the following genera: *SWB02* (23.18%), unassigned (22.01%), *SM1A02* (18.80%), *Ferrovibrio* (13.72%), and *Acinetobacter* (7.88%) (Figure 4.21).

The microbial composition of the river water filtrate sample indicated that *Arcobacter* (34.08%) was the most dominant genus, while the unclassified genera consisted of 64.10% of the total microbial community. The wastewater filtrate sample was dominated by the following genera: *SM1A02* (13.93%), *Arcobacter* (13.13%), *SWB02* (10.27%), and *Methyloversatilis* (8.35%). The unassigned genera consisted of 42.05% of the total microbial community found within the wastewater filtrate sample. The biofilm surrounding the wood substrate consisted majority of unassigned genera (59.28%) followed by *Roseomonas* (10.66%), *Sphingomonas* (8.65%), and *SWB02* (7.05%) (Figure 4.21).

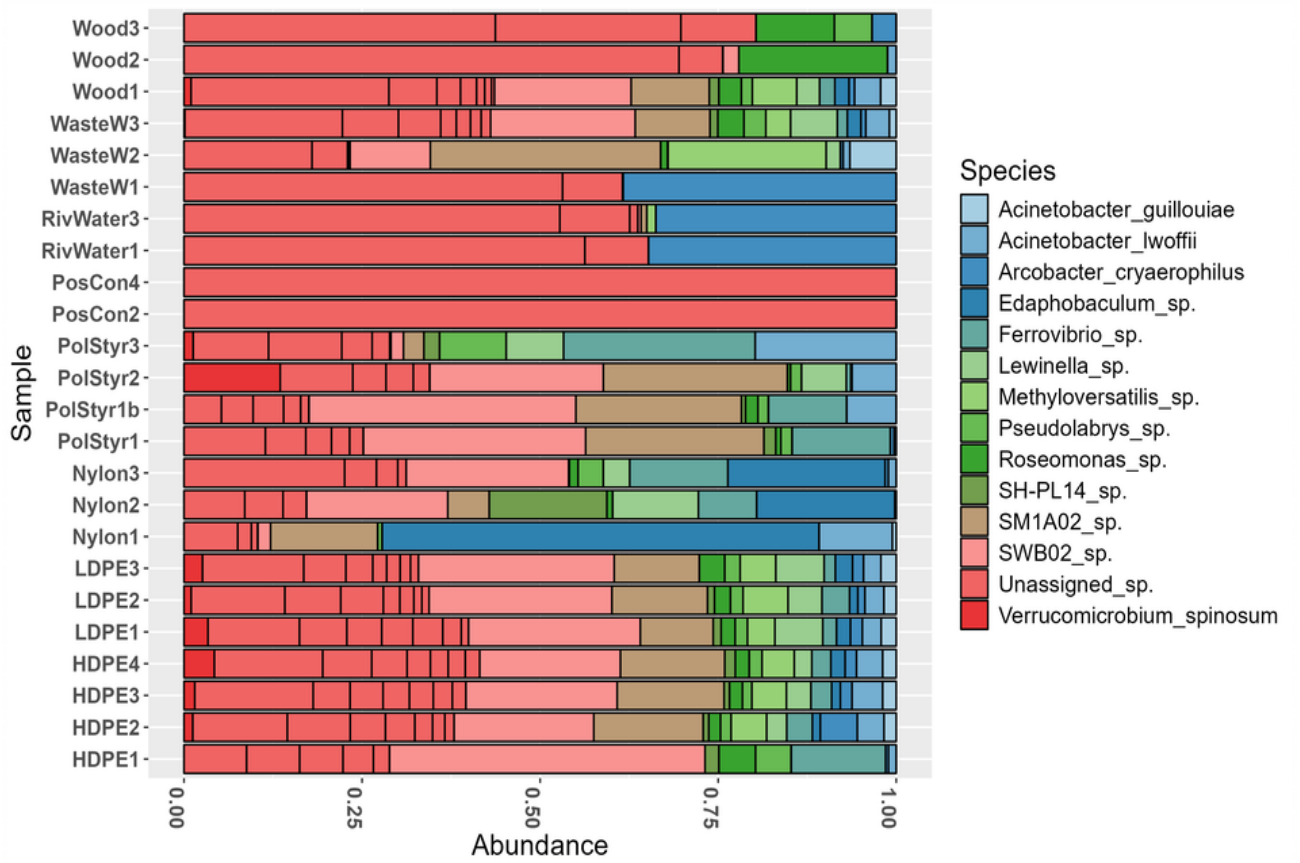


Figure 4.22: Relative abundance of microbial colonisation as different species.

Figure 4.22 illustrates the different species found within the bacterial community surrounding the microplastics. 13 bacterial species were found, namely, *Acinetobacter guillouiae, Acinetobacter lwoffii*, *Acrobacter cryaerophilus, Edaphobaculum* sp., *Ferrovibrio* sp., *Lewinella* sp., *Methyloversatilis* sp., *Pseudolabrys* sp., *Roseomonas* sp., *SH-PL14* sp., *SM1A02* sp., *SWB02* sp., *Verrucomicrobium spinosum* and unassigned species. The most common species found within the biofilm that surrounded the microplastics include *SWB02*, *SM1A02*, *Edaphobaculum* sp. and *Ferrovibrio* sp. *SWB02* sp. was the most dominant species found in the biofilm surrounding the HDPE, LDPE, nylon 2, nylon 3, PS1, PS1b and PS2 substrates, *SM1A02* sp. was dominant in the wastewater 2 biofilm, *Edaphobaculum* sp. in the nylon 1 Biofilm and *Ferrovibrio* sp. in the PS 3 biofilm. Unassigned species were detected at positive controls 2 and 4 (100%).

The most dominant bacteria found within the HDPE plastisphere are, *SWB02* sp. (26.23%), *SM1A02* sp. (11.25%), and *Ferrovibrio* sp. (5.61%). The majority (35.21%) of the microbial community surrounding the HDPE polymer could not be identified. The species detected in the LDPE biofilm is as follows: Unassigned sp. (33.43%), *SWB02* sp. (25.77%), *SM1A02* sp. (11.86%), *Lewinella* sp. (6.09%), and *Methyloversatilis* sp. (5.06%). The nylon plastisphere was dominated by the following bacterial species: *Edaphobaculum* sp. (34.28%), unassigned

sp. (19.58%), *SWB02* sp. (14.82%), *Ferrovibrio* sp. (7.31%), and *SM1A02* sp. (6.97%). The HDPS polymer was dominated by the following species: *SWB02* sp. (23.71%), *SM1A02* sp. (19.25%), *Ferrovibrio* sp. (13.07%), and *Acinetobacter Iwoffii* (8.32%). A portion of the microbial community consisted of unassigned species (22.86%) (Figure 4.22).

The dominant species within the river water filtrate is *Arcobacter cryaerophilus* (34.28%), while a large portion of unassigned species were detected (64.46%). The wastewater filtrate sample had the following dominant species: *SM1A02* sp. (14.29%), *Arcobacter cryaerophilus* (13.14%), *SWB02* sp. (10.53%), and *Methyloversatilis* sp. (8.56%). A portion of unassigned species were also detected (42.60%). The biofilm surrounding the wood substrates was dominated by the following bacterial species: *Roseomonas* sp. (11.68%), and *SWB02* sp. (7.14%), whilst a large portion of the microbial community was unassigned (66.18%) (Figure 4.22).

4.12. Summary of results

The results in this study contained microcosm experiments for the classification of the 'Plastisphere' in a simulated South African river system. Two water samples were taken at the start of the microcosm study, where one sample was river water, whilst the other was WWTP effluent after chlorination but before being released into the environment, obtained from the WWTP in Potchefstroom, NWP, SA. Scanning electron microscopy images demonstrated the attachment of bacterial communities, once introduced to the water source and incubation occurred. The SEM images illustrated microbial attachment and colonisation after a 28-day incubation period. The results indicated that biofilm formation occurs more readily in high-nutrient environments (WWTP effluent). It can be seen that the microbial colonisation was influenced by the surface of the substrates, where, if the substrate's surface was uniform, the microbial colonisation decreased. Wood had an uneven surface pre-incubation and post-incubation, and the microbial attachment was, therefore, higher.

Ten different microplastics were collected on different days during the incubation period for the investigation of bacterial growth. A total of 877 bacterial isolates were collected through plating techniques onto R2A agar to provide to best possible chance of bacterial growth. The isolate amounts were lessened through various identification techniques, such as extracellular enzymatic testing and gram staining. The extracellular enzymatic test indicated that 49.3% tested positive for the presence of haemolysis, 32.6% for the presence of proteinase, 27.7% for DNase and lastly 38.5% for the presence of lipase.

The Kirby Bauer disc diffusion method was used on 51 bacterial isolates that presented a high virulence to determine the antibiotic resistance of the isolates. 41.1% of the microbial isolates were resistant to V30. 78.4% were resistant to A10, 13.8% to K30 µg, 11.7% to C30 and 64.7% were resistant to T5. Amoxicillin is determined to be the least effective antibiotic used for determining the ABR of the bacteria, whereas chloramphenicol is the most effective antibiotic.

Gram staining was performed on the 51 bacterial isolates to aid with identification purposes and provide information regarding the cell structure. The majority of the bacterial isolates could be described as gram-negative, with one isolate presenting as Gram-positive.

Antibiotic resistance profiles and various virulence characteristics were used to generate a PPI, to indicate the virulence potential of the isolates obtained. From the PPI, 16 bacterial isolates were chosen for further identification through Sanger sequencing.

Sanger sequencing led to the construction of a maximum likelihood phylogenetic tree whereby bootstrapping was performed to gain accuracy. Bacterial isolates identified include, *Aeromonas hydrophilia*, *Aeromonas caviae*, *Aeromonas veronii*, *Providencia vermicola*,

Providencia rettgeri, *Stenotrophomonas koreensis*, *Brevundimonas diminuta*, and *Bacillus subtilis*.

Biofilm profiling led to the determination of the Alpha- and Beta diversity found in the plastisphere. The Alpha diversity of the bacterial community composition demonstrated the highest dominance detected at the wood substrate compared to river water which had the highest diversity and richness. These results illustrate the difference in microbial community on substrates and water sources. Beta Diversity of the isolated samples was illustrated by the Jaccard similarity index. The Jaccard similarity index indicated that the majority of the substrates shared the same community composition, except for wastewater and wood. The nMDS plot indicated an overlap between the communities found at HDPS, HDPE, nylon and LDPE while the positive control, river water and wood formed more discrete groups.

Next-generation sequencing led to the identification of five dominant phyla, Proteobacteria, Planctomycetota, Cyanobacteria, Patescibacteria and Bacteroidota. 14 different genera, *Acinetobacter, Acrobacter, Edaphobaculum, Ferrovibrio, Lewinella, Methyloversatilis, Pseudolabrys, Roseomonas, SH-PL14, SM1A02, Sphingomonas, SWB02, Verrucomicrobium* and unassigned genus, 13 species were identified, namely, *Acinetobacter guillouiae, Acinetobacter Iwoffii*, *Acrobacter cryaerophilus, Edaphobaculum* sp*., Ferrovibrio* sp*., Lewinella* sp*., Methyloversatilis* sp*., Pseudolabrys* sp*., Roseomonas* sp*., SH-PL14* sp*., SM1A02* sp*., SWB02* sp*., Verrucomicrobium spinosum* and unassigned species. Positive controls 2 and 4 demonstrated 100% unassigned microbial composition.

Aeromonas hydrophilia and *Aeromonas caviae* were isolated from the HDPS and LDPE biofilms while *Aeromonas veronii* and *Providencia vermicola* were isolated from the HDPE biofilm. *Bacillus subtilis* and *Providencia rettgeri* was isolated from the wood biofilm. The isolation of the bacteria on the nylon biofilm resulted in the identification of *Stenotrophomonas koreensis* compared to the identification of *Brevundimonas diminuta* from the wood and HDPS biofilm.

Aeromonas sp., *Providencia* sp. and *Stenotrophomonas koreensis* belong to the Gammaproteobacteria class (Seshadri *et al*., 2006; Fluit *et al.,* 2022; Idomir, 2021). The Gammaproteobacteria class was observed at the majority of the samples except for the positive control samples. *Brevundimonas diminuta* belongs to the Alphaproteobacteria class and was detected in the majority of the samples except for the positive control samples, river water 1, and wastewater 1 sample (Ryan & Pembroke, 2018). *Bacillus subtilis* belongs to the Firmicutes phylum as observed above. The various substrates indicated the presence of pathogenic bacteria, while NGS revealed that the classes and phyla detected support the findings of the bacteria isolated.

Next-generation sequencing revealed 13 isolated species compared to the 8 species detected through Sanger sequencing. The difference in species identified can be attributed to the restrictions encountered by culture-dependent techniques while NGS provides an unbridled insight into the bacterial composition within an environment.

Chapter 5 : Discussion

This chapter focuses on the interpretation of the results obtained in Chapter 4 to mitigate the potential threat of micro-plastics in freshwater environments by providing an insight into the vectorial probability of micro-plastics for the transportation of micro-organisms and, in doing so, the transportation of ARB in freshwater systems. Thus, this study aims to provide insight into microplastic -micro-organism interactions in a river system in the NWP in SA.

5.1. Microcosm experiments: Cell colonisation on microplastics and wood particles

Microplastics are considered factitious substrates for colonisation and possible vectors for transporting microbial assemblages within aquatic environments (Lambert & Wagner, 2018, Miao *et al*., 2019). The bacterial assemblages exchange genetic information through different methods, such as transduction, transformation, and conjugation (Koraimann & Wagner, 2014). Horizontal gene transfer can also occur within biofilms across the different species. This study aimed to investigate the microbial assemblages on sterile microplastics and wood substrates when introduced to a combination of river water and WWTP effluent.

Scanning electron microscopy images (Figure 4.1 - Figure 4.6) revealed that microbial colonisation occurred within a 28-day period. Notably, microbial attachment was more pronounced in the grooves, pits, and uneven edges, as in the case of the wood substrates. This is seen as consistent with past research studies, which indicated that the microplastic surface properties played a role in microbial attachment and survival (Mah, 2012). The SEM images of this study showed that microbial colonisation was made possible by fimbria extension on the microplastics and wood substrate. Whilst the substrate surfaces guide the microbial attachment, the morphological structures, along with surrounding environmental conditions, can also influence microbial colonisation and attachment capabilities (Lambert & Wagner, 2018, Miao *et al*., 2019).

In examining the different mediums - microplastics and source water (Figure 4.2 - Figure 4.3) - substrates incubated in a mixture of river water and wastewater were less colonised than the positive control containing only WWTP effluent. Wastewater treatment plant effluent contains a greater nutrient concentration compared to what is found in river water, which increases microbial colonisation (Ziajahromi *et al*., 2016; Wang *et al*., 2015). The elevated nutrient concentration in WWTPe can be attributed to the inadequately functioning WWTPs in SA. The Green Drop report for 2022 indicated that within the NWP, the WWTPs are in a critical state,

with numerous pumps out of order and the capacity to treat sludge was diminished, resulting in an increased nutrient load (DWA, 2022).

Therefore, with the increased nutrient load, the microbial colonisation was greater compared to the microcosms containing a mixture of sterilised river water and WWTP effluent, due to the dilution effect the river water had on the WWTP effluent and may explain the formation of biofilms as early as day 3. Furthermore, the formation of aggregates was more prevalent in the microcosms containing wood particles (Figure 4.6). The negative control had no microbial growth, indicating the absence of bacterial contamination throughout the incubation period.

In an environmental context, elevated nutrient levels due to increased anthropogenic activity and improper disposal of waste, and microplastic aggregation due to biofilm formation may pose health risks to animals and humans within the surrounding area (Wang *et al*., 2015). There is a knowledge gap regarding the animal–microplastic interaction and, therefore, the capability to transfer pathogenic bacteria to the animals. Toyofuku *et al.* (2015) reported that environmental cues, such as high nutrient load and favourable environmental conditions, can accelerate primary and secondary biofilm development without being substrate specific. Scanning electron microscopy images of day 3 support the findings of Toyofuku *et al.* (2015).

Scanning electron microscopy analysis revealed that the surface structure played a role in the microbial cell attachment leading to a variety of bacterial cells on the different microplastic substrates. By day 28, distinct biofilm formations were noted across the different substrates (Figure 4.2). In the positive control microcosm, the surface of the HDPE polymers exhibited a slime barrier, resulting from the overpopulation of microbes and the surrounding aggregates, which made it difficult to visualise the morphology of the cells (Figure 4.3). The slime barrier was potentially induced by the clustering and adherence of the microbes driven by the high nutrient load, as well as environmental factors, such as temperature and movement, combined with the pre-existing bacteria within the WWTP effluent. The wood substrates used in this study were representative of naturally occurring particles within an aquatic environment. The microbial colonisation was all the more evident on the wood substrate than on the microplastic substrates in the river water and WWTP effluent mixture (Figure 4.6). The increased surface ratio caused by folds and compartments of the wood particles resulted in increased microbial attachment and biofilm formation. The microplastics used in this study, except for HDPS polymer, had smooth surfaces. The HDPS polymer, with its uneven foam-like structure, resulted in an increased microbial cell attachment in the pit and grooves of the substrate (Figure 4.4).

Data obtained from the DNA extraction of the various substrates (microplastics and wood) indicated that the wood substrate had the highest degree of bacterial DNA extracted after the 28-day incubation, corresponding with the obtained SEM images (Figure 4.6 and Figure 4.7). The LDPE polymer presented the least amount of bacterial colonisation as presented by the total eDNA extracted (see Figure 4.7).

5.2. Virulence testing

Enzymes are required at all stages of life for the continuous functioning of cellular processes along with the continuous breakdown of substrates. Extracellular enzymes are considered one of the leading procedures for quick partial identification of unknown bacterial origin through the use of selective and differential media (Loperena *et al*., 2012). Extracellular enzyme testing has been successfully used in the novel identification of environmental isolate activities through semi-quantitative determinations, the halo size and growth of the colony (Loperena *et al*., 2012). Loperena *et al*. (2012) found that the most frequent enzymatic activity among bacterial isolates includes lipase, DNase, amylase, caseinase and gelatinase. Previous studies investigated the production of extracellular enzymes due to their role in pathogenesis (Loperena *et al*., 2012, Pavlov *et al*., 2004)

In this study, 877 bacterial isolates were cultured and underwent extracellular enzyme testing. Isolates that tested positive for one or more extracellular enzymes were used for further identification. The results are presented in Figure 4.8. As seen in Table 4.1, the numerical value awarded depended on the isolate's capabilities to produce certain enzymes, where 49.23 % tested positive for the presence of haemolysis and 32.6% for proteinase. The overall trend in the percentage of isolates that produced the extracellular enzymes is as follows: haemolysins (49.23%), lipase (38.5%), proteinase (32.6%), and lastly, DNase (27.7%). The majority of these enzymes are associated with virulence screening, where their presence may indicate the pathogenic potential of an organism (Georgescu *et al*., 2016).

Out of the 388 isolates that displayed haemolysins, 51 isolates (13.14%) produced more than three enzymes and are therefore regarded as potentially pathogenic (Edberg *et al*., 1996). Results for the unidentified isolates indicate the presence of several enzymatic enzymes that can contribute towards the virulence of the isolated bacteria Thereafter, the extracellular enzymatic results were combined with the antibiotic susceptibility test results to determine the pathogenic potential of the isolated microbes.

5.3. Antibiotic susceptibility

Antibiotic resistance in bacterial pathogens has become a worldwide challenge associated with high morbidity and mortality combined with ineffective antimicrobial agents (Frieri *et al*., 2017). Due to the overuse of antibiotics, bacteria make use of various mechanisms to acquire antibiotic-resistance genes and in doing so, render antibiotic therapy ineffective (Zhang *et al*., 2009). It has become apparent that acquired resistance poses a threat to the proper functioning of antibiotics. Due to the widespread and unregulated use of antibiotics in the environment, agricultural, and clinical settings, which in turn results in the emergence of antibiotic reservoirs. Within these environments, resistance genes are disseminated by HGT (Nhinh *et al*., 2021), reaching human pathogens or antibiotic-resistant pathogens in the environment. Antibiotic resistance is described as the micro-organism's capacity to survive and grow at increased antibiotic concentrations for extended periods and is determined by assessing the minimum inhibitory concentration (MIC) in contrast to antibiotic tolerance, which is seen as the capacity of bacteria to survive a transient exposure to increased antibiotic concentrations and assessed by the minimum bactericidal concentration needed to eliminate 99% of the cells (Uruén *et al*., 2020).

In this study, the antibiotic resistance, susceptibility, or intermediate resistance of bacterial isolates was tested in order to determine the potential pathogenicity of the bacterium surrounding the microplastics. The Kirby Bauer disc diffusion method was used with VA30, A10, T5, K30 and C30 due to the studied overuse and abundance within the aquatic environment (Flores-Vargas *et al*., 2021). The varying degrees of antibiotic resistance across the different isolates could be a result of HGT within the various biofilms. The bacterial isolates presented the highest resistance towards A10, followed by T5; while the isolates presented the least resistance towards C30 and K30, while intermediate resistance was observed towards VA30. (Table 4.2).

Amoxicillin is a broad spectrum, β-Lactam antibiotic and can be described as an acid-stable, semi-synthetic drug belonging to the Penicillin class with the ability to be effective against a range of infections caused by Gram-negative or Gram-positive bacteria (Kaur *et al*., 2011; Meng *et al*., 2017). A large portion of the isolates (78.4%) presented resistance or intermediate resistance towards A10. Trimethoprim belongs to the Sulphonamide class and diaminopyrimidine group of compounds and has been in use for decades due to being an efficient and inexpensive antibacterial agent used in both humans and animals (Sköld, O. 2001). Trimethoprim only interferes with bacterial cells, which leads to frequent use and the acquired resistance thereof, due to the various transfer mechanisms and survival mechanisms

in and between bacteria (Sköld, O. 2001). The majority of the bacterial isolates presented varying degrees of resistance towards T5 in the current study, which aligns with the results presented by Mulamattathil *et al.* (2014) with regards to isolates obtained from surface and drinking waters.

Biyela *et al.* (2004), Islam *et al.* (2010), and Zhang *et al.* (2020) found that isolates obtained from microplastics from aquatic environments were highly susceptible towards C30 (80%); the current results indicate that C30 presented the highest susceptibility (88.3%), therefore supporting their findings. Kanamycin is an antibiotic used to treat serious bacterial infections (Zhang *et al*., 2019). France *et al*. (2019) studied the antibiotic-resistant mutation accumulation in biofilm and found that kanamycin-resistant mutants increased with the introduction of K30 to the biofilms. The current study found that the bacterial isolates isolated were the least resistant towards K30 compared to the other antibiotics.

Vancomycin forms part of the tricyclic glycopeptide class and is seen as an active agent against Gram-positive aerobic cocci and bacilli (Nhinh *et al*., 2021; Rubenstein & Keynan, 2014). The killing effect of VA30 is characterised by a slow mode of action, hampered by the large bacterial inoculum, stationary growth phase and aerobic conditions as a result of the weakening of the bacterial cell wall and, ultimately, the leakage of intracellular components (Alvarez *et al*., 2016). A few of the pure isolates indicated resistance and intermediate resistance towards V10 (41%) (Table 4.2).

Pharmaceutical compounds are released mostly unchanged or as metabolites and diluted due to the large water volumes in WWTP's (Ngqwala & Muchesa, 2020). Pharmaceuticals commonly detected in South African surface water sources include analgesics, antibiotics, anti-inflammatories and antiretrovirals (Ngqwala & Muchesa, 2020). Kanama *et al*. (2018) studied the occurrence of pharmaceuticals in WWTP in the North West province in SA, they found that due to the inadequately treated effluents that are being released into the environment, C30 detection was low, while ibuprofen was the dominant pharmaceutical detected in the WWTP effluent. Trimethoprim has also been detected in water originally released from WWTP in Durban, SA (Matongo *et al*., 2015). Trimethoprim was also detected in the Umgeni River, and Msundizi tributary with a concentration of 0.85 µg/L (Matongo *et al*., 2015).

Culture-based methods are less sensitive but more cost-effective, replicable and widely accessible (Stevenson *et al*., 2023). Limitations with culture-based and culture-independent (PCR) methods include the inability to culture unknown microbes due to the unknown growth conditions and the absence of prior knowledge of microbes for culture-independent methods

such as polymerase chain reaction (Boers *et al*., 2019). New culture-independent methods are needed to improve antimicrobial stewardship, the detection of viable but non-culturable microbes and investigating unknown pathogens (Boers *et al*., 2019).

5.4. Bacterial isolates

The isolates obtained during this study had various characteristics associated with virulence that may contribute to their pathogenic potential. Therefore, the results obtained from the extracellular enzyme production and antibiotic resistance were combined to generate a PPI. The goal of this index is to determine the degree of pathogenicity and disease-causing possibility (Svetlicic *et al*., 2023).

Pathogenic bacteria can survive and grow in different environments, such as surface waters, groundwater and WWTP, depending on the available nutrients within the environment (Vital *et al*., 2010). Table 4.3 demonstrates the pathogenic potential of each isolate that presented more than two extracellular enzyme products and the antibiotic resistance of each isolate. The pathogenic potential of these isolates demonstrates the risk associated with bacterial attachment to microplastics and the occurrence of ARB within these biofilms (Rodriguez-Mozaz *et al*., 2015).

South Africa experiences water shortages due to improper equipment and management, and in rural settlements, the absence of proper water systems and inadequate water supplies results in the use of rivers and streams for household duties (Dzoyem *et al*., 2016). Due to the unsafe waters used, an increased risk of infection through water-borne pathogenic bacteria is possible (Arnone & Walling, 2007). The presence of potentially pathogenic bacteria within a simulated freshwater environment, such as in this current study, demonstrates the urgency and need for WWTP's within the NWP to be better equipped and maintained to decrease the ever-growing threat to the finite freshwater systems due to the release of WWTP effluent within the environment. Therefore, the potentially pathogenic bacteria isolated can be introduced to the receiving environment, whereby various anthropogenic sources will result in a greater resistance towards common antibiotics (Faleye *et al*., 2019). This, in turn, will result in antibiotics within the agricultural and health sectors being used more readily to combat ARB, thereby engaging in cyclic behaviour (Rodriguez-Mozaz *et al*., 2015).

The 51 isolates that tested positive for numerous enzymatic tests and antibiotic susceptibility resulted in 16 isolates demonstrating pathogenic potential. These 16 isolates were isolated from the different microcosms. The HDPS plastisphere contained the most potentially pathogenic bacteria (7), and the wood biofilm had the second-most potentially pathogenic

bacteria (3). At the same time, the nylon, HDPE and LDPE presented the least amount of potentially pathogenic bacteria within their respective biofilms (2 each) (Figure 4.11). McCormick *et al*. (2016) hypothesised that there will be discrepancies between various plastispheres derived from microcosm studies due to plastic composition, location within the water column, buoyancy, size and density.

Of the 16 isolates identified through 16S rRNA Sanger sequencing, *Aeromonas hydrophila, Aeromonas caviae, Stenotrophomonas koreensis, Bacillus subtilis, Brevundimonas diminuta, Providencia vermicola, Aeromonas veronii and Providencia rettgeri* were identified. Therefore, the discussion focuses on the origin and possible pathogenicity of the species identified.

5.4.1. *Aeromonas* sp.

As illustrated in Table 4.4, 56.3% of the identified isolates could be classified as *Aeromonas* spp. Most of the *Aeromonas* spp. isolated originated from the HDPS and LDPE biofilms. According to Seshadri *et al*. (2006), *Aeromonas* spp. are found in a variety of aquatic environments with warmer climates, along with the capability to cause infections in vertebrates and invertebrates and can be described as facultative anaerobic chemo-organotrophs capable of anaerobic respiration and dissimilatory metal reduction along with certain virulence factors, such as haemolysis and proteinases and represents a group of motile mesophilic species. *Aeromonas* spp. are found in the Aeromonadaceae family within the gamma-proteobacteria class (Seshadri *et al*., 2006).

The *Aeromonas* genus can be divided into two phenotypically distinct groups, psychrophilic and non-motile, *A. salmonicida*, and mesophilic, motile and heterologous, which include *Aeromonas hydrophila*, and *Aeromonas caviae* (Erdem *et al*., 2008). *Aeromonas* sp. is known as an opportunistic and pathogenic bacterium with human infections, including gastrointestinal tract syndromes, wound and soft tissue infections and various other infections such as respiratory tract, sepsis and eye infections (Ottaviani *et al*., 2011; Igbinosa *et al.,* 2017). Nascimento *et al*. (2024) found that *Aeromonas* spp. could be seen as strong biofilm producers, combined with the ability to lyse red blood cells, resulting in virulence factor consideration. *Aeromonas* sp. can express a wide variety of virulence factors, involved in biofilm formation, cell adherence, invasion and cytotoxicity, including adhesins, extracellular toxins, and enzymes (Talagrand-Reboul *et al*., 2020).

Multidrug resistance has been reported for *Aeromonas* spp. isolated from fish and various water sources, such as rivers, WWTP effluent and lakes (Nascimento *et al*., 2024). The harbouring of antibiotic-resistance genes obtained for WWTP, for instance, has led to these

organisms being considered ecological indicators for water pollution (Nascimento *et al*., 2024). Antibiotic resistance amongst *Aeromonas* spp. can be attributed to intrinsic resistance, specific gene mutations, efflux pumps and HGT mechanisms (Nascimento *et al*., 2024).

A. hydrophila, *A. caviae,* and *A. veronii* are most known to cause intestinal and extraintestinal infections in humans (Ottaviani *et al*., 2011; Igbinosa *et al*., 2017). *A. hydrophila* poses a singular polar flagellum as well as the ability to counteract antimicrobial factors, such as β-lactamases, C30 acetyltransferases and other proteins that confer resistance towards bicyclomycin and aminoglycosides (Seshadri *et al*., 2006). *A. veronii* was identified as a strong biofilm producer that is resistant towards different antibiotics (Nascimento *et al*., 2024). *Aeromonas hydrophila* attachment towards PS, PVC and PE has been documented (Igbinosa *et al*., 2017) and supports this study's findings.

A study was done to determine the virulence of 12 different species of *Aeromonas*; the study found that *A. jandaei* ATCC 49587 was the most pathogenic, followed by *A. hydrophila* ATCC 7966 (Seshadri *et al*., 2006). Sadar *et al*. (2016) and Nhinh *et al*. (2021), found that *A. hydrophila* were resistant to A10 and VA30, and partially resistant to tetracycline and K30. *A. hydrophila* possessed several virulence factors, as illustrated in Table 4.3. The heterogeneity of the virulence found in *A. hydrophila* isolates in present and previous studies demonstrates the variability of virulence profiles of the isolates and the pathogenicity thereof (Nhinh *et al*., 2021). Sader *et al.* (2016) and Seshadri *et al* (2016) validate the study's finding about *A. hydrophila* and the pathogenic potential it poses.

A. caviae was also identified during this study. Rocha-de-Souza *et al*. (2008) found that an increased percentage of *A. caviae* attaches to and invades host cell lines compared to *A. hydrophila* and may contain some of the same antibiotic resistance properties. According to Abbot *et al*. (2003) and Nhinh *et al*. (2021), the ever-growing expansion of the genus *Aeromonas* influences the ability to properly identify the different species within the genus combined with the heterogeneous nature they pose. The current study revealed that 37.5% of the identified isolates were classified as *Aeromonas hydrophila* species strain ATCC 7966.

The isolated *Aeromonas* spp. exhibited resistance primarily to A10, while VA30 and T5 resistance was also observed, indicating that a multidrug-resistant *Aeromonas* spp. was present in the simulated freshwater environment (Table 4.5). Chloramphenicol resistance was the least detected across the different *Aeromonas* spp. isolated. The *Aeromonas* spp. isolated produced proteinase, DNase, and lipase with varying degrees of haemolysis, corroborating the findings of Nascimento *et al*. (2024) and Monfort and Baleux (1991).

5.4.2. *Stenotrophomonas koreensis*

The *Stenotrophomonas koreensis* strain TR6-01 was identified as one of the isolates originating from the nylon biofilm. The genus *Stenotrophomonas* comprises 16 species, namely *Stenotrophomonas acidaminiphila*, *S. bentonitica, S. maltophilia, S. nitrriducens, S. koreensis, S. humi, S. terrae, S. ginsengisoli, S.* sp*. D-1, S. chelatiphaga, S. daejeonensis, S. pavanii, S. tumulicola, S.* sp*. DDT-1, S dokdonensis and S. rhizophila* and belongs to the class Gammaproteobacteria, and the Family Xanthomonadaceae (Yang *et al*., 2006, Lee *et al*., 2011, Wang *et al*., 2018; Fluit *et al.,* 2022). The cells are Gram-negative, aerobic, non-motile, non-spore-forming rods, capable of producing proteinase, haemolysins, and lipase along with offering resistance towards K30 and A10 (Yang *et al*., 2006; Ryan *et al*., 2009). Species of the genus *Stenotrophomonas* are found in a wide variety of environments, including soil and sewage water and are highly adaptable to hostile and nutrient-limited environments, such as river water (Lee *et al*., 2011, Wang *et al*., 2018; Ryan *et al*., 2009). *Stenotrophomonas* can form fimbria or filli to attach and colonise surfaces and aid in biofilm formation (Fluit *et al*., 2022).

Fluit *et al*. (2022) found that infections caused by *Stenotrophomonas* are located in the airways and give rise to nosocomial infections and lung disease. The CLSI has not defined breakpoints for *Stenotrophomonas* spp. (Wang *et al*., 2018), therefore, the average resistance measurement for T5, A10, K30, C30, and VA30 was used to determine the antibiotic resistance of the isolated species. *Stenotrophomonas* spp. exhibited resistance primarily to VA30, K30 and T5 while A10 and C30 demonstrated varying degrees of resistance. Li *et al* (2009) isolated *Stenotrophomonas koreensis* in penicillin production WWTP effluent and downstream of the released WWTP effluent and found varying degrees of co-resistance towards A10, C30, and K30 compared to the decreased resistance towards VA30. The current study is supported by the findings of Li *et al*. (2009) and Deng *et al*. (2022) findings. β-haemolysis was observed along with the production of proteinase, lipase and DNase during the current study (Table 4.5). Yang *et al*. (2006) isolated *S. koreensis* from river water near compost fields and found that it degrades DNA and produces protease as well as being susceptible to K30.

5.4.3. *Bacillus subtilis*

Bacillus subtilis is found in a myriad of different environments, including soil and aquatic environments, but they are also known for forming surface-associated multicellular aggregates, such as biofilms (Earl *et al*., 2008). *Bacillus subtilis* was isolated from the wood biofilm.

Bacillus subtilis is a Gram-positive, rod-shaped, endospore-forming bacteria belonging to the Bacillaceae family, along with being the most studied species in the genus *Bacillus* due to its capability for acquiring extracellular DNA that facilitates genetic modification and occurrence of sporulation along with the possibility to produce lipase and proteinase combined with the absence of toxic byproduct (Kovács A.T. 2019; Aguilar *et al*., 2007; van Dijl & Hecker, 2013).

Gu *et al*. (2019) and Paes *et al.* (2012) found that *Bacillus subtilis* isolated from wastewater produces β-haemolysin and proteinase*,* which supports this study's findings with the enzymes produced. Lipase and DNase were also produced and are in line with the findings of Ehling-Schulz *et al*. (2019). The ability of *Bacillus* sp. to form biofilms in aquatic environments (Veysseyre *et al*., 2015) can, therefore, be seen as problematic due to the endospore formation, acquisition of extracellular DNA, extracellular enzyme production and the acquired antibiotic resistance as demonstrated by this study. The current study found that *Bacillus* spp. isolated from the wood biofilm exhibited resistance towards a variety of antibiotics, such as A10, VA30, K30, and T5 resistance being the most prevalent, along with presenting intermediate resistance towards C30. Hammuel *et al.* (2023) found that *Bacillus subtilis* isolated from cooked meat presented resistance towards C30 and A10. Even though Hammuel *et al.* (2023) found multidrug-resistant *Bacillus* in food, the current study found multidrug-resistant *Bacillus subtilis* in a simulated freshwater environment, indicating the presence of pathogenic *Bacillus* in multiple environments.

5.4.4. *Providencia* sp*.*

The *Providencia* genus is found in the Enterobacteriaceae family, within the Enterobacteria order and as part of the Gammaproteobacteria class (Idomir, 2021). Micro-organisms of the *Providencia* genus are Gram-negative bacilli, unencapsulated, mobile, non-sporogenic and aerobic bacteria, they can be found in aquatic environments along with presenting pathogenic towards animals (Idomir, 2021). *Providencia* sp*.* is seen as commensal flora within the gastrointestinal tract (Yoh *et al*., 2005). *Providencia vermicola* belongs to the genus Providencia, the family, Morganellaceae and the order Enterobacterales (Lupande-Mwenebitu *et al*., 2021; Somvanshi *et al*., 2006) The deamination of specific amino acids through oxidation into the corresponding keto and ammonia acids, aids in differentiating *Providencia* sp. from other members of the Enterobacterales family (Lupande-Mwenebitu *et al*., 2021). The current study found that *P. vermicola* was resistant towards VA30, A10, C30, and T5, which is supported by Lupande-Mwenebitu *et al*. (2021) findings.

Providencia rettgeri is found in surface waters, sewage, reptile, and amphibian faeces (Sharma *et al*., 2017) and is found to be resistant towards VA30, A10 and T5 (Lupande-

Mwenebitu *et al*., 2021). Yoh *et al.* (2005) and Idomir (2021) found that *P. rettgeri* can cause diarrhoea, abdominal pain, urinary tract infections, meningitis, endocarditis, and bloodstream infections.

The *Providencia* spp. isolated exhibited a multitude of extracellular enzymes, such as β-haemolysins, proteinase, DNase, and lipase. In the current study, *Providencia* spp. isolated from the HDPE and wood biofilms exhibited resistance to a variety of antibiotics, VA30, A10 and T5 resistance being most prevalent, but varying levels of resistance to K30 and C30. *Providencia vermicola* is seen to be susceptible towards K30.

5.4.5. *Brevundimonas diminuta*

Brevundimonas diminuta can be found in different environments, such as soil, sea sediment, sludge, and aquatic environments, with resistance to a wide array of antimicrobials, they are Gram-negative, aerobic, non-fermenting bacteria belonging to the Alphaproteobacteria class and the Caulobacteraceae family (Ryan & Pembroke, 2018, Han & Andrade, 2005). *Brevundimonas diminuta* has been isolated from clinical specimens, including blood and urine with a decreased virulence, or it can be used as a test organism for reverse-osmosis (RO) filters and testing the porosity of pharmaceutical-grade filters (0.2 µM) but has been observed to penetrate these filters (Ryan & Pembroke, 2018). Ryan and Pembroke (2018) found that *Brevundimonas* infections occur more commonly when a person's immune system is compromised by an underlying condition, such as urinary tract infections and cancer and can, therefore, be seen as an opportunistic pathogenic bacterium.

Chi *et al.* (2004), Swain and Route (2017) and Arafat *et al.* (2023) found that *Brevundimonas diminuta* isolated from HDPS biofilms in estuaries, indicated resistance towards T5, K30, and A10 while antibiotic susceptibility was recorded at VA30. The current study found that *B. diminuta* isolated from HDPS and wood biofilms presented resistance towards A10, C30, T5, and K30, whilst C30 presented varying degrees of resistance. Arafat *et al.* (2023) also found that *B. diminuta* presented the ability to hydrolyse skim milk (protease) compared to the protease, lipase, and DNase recorded during the current study.

5.5. Phylogenetic analysis

Figure 4.14 illustrates the relationship between the different isolates. Sequences with high similarities (≥94%) were used to construct a Maximum likelihood phylogenetic tree (Figure 4.14) using MEGA 11 (Tamura *et al*., 2021). The phylogenetic tree illustrates the relationship of the 9 bacterial species detected with a limited degree of evolutionary change, indicating a common ancestor.

A neighbour-joining phylogenetic tree is used based on the distance between the species, whereas the maximum likelihood phylogenetic tree is used to determine the evolution of the species (Hong *et al*., 2020; Tillier & Collins, 1995). The maximum likelihood method is highly robust towards the assumption that sites evolve independently compared to the neighbour-joining method, which proves more sensitive (Tillier & Collins, 1995). The Maximum likelihood method is frequently used due to its flexibility, computational tractability, and complex evolutionary models (Douady *et al*., 2012; Hordijk & Gascuel, 2005). Maximum likelihood phylogenetic trees have been used in various studies that analysed microbial communities in WWTP effluent and river water (Zhang *et al*., 2009; Gambarini *et al*., 2021; Hordijk & Gascuel, 2005; Silva *et al*., 2017).

In Figure 4.14, the evolutionary relationship of the 16SrRNA gene sequences from the bacterium isolates can be observed. The tree is rooted with *Escherichia coli* as the outgroup, providing a reference point for interpreting the relationships among the other taxa. Species of the same genus clustered together based on sequence similarities, with the support of strong bootstrap values (100%). Single clusters formed between the selected bacterial groups. These groupings were supported with strong bootstrap support (above 95%).

Aeromonas caviae strain CECT 4221 and *Aeromonas caviae* strain 15468 are clustered together with a high bootstrap support of 99%, indicating the evolutionary relatedness between these bacteria. This suggests a common ancestor between these strains. These strains form a subgroup of *Aeromonas hydrophila* strain ATCC 7966 and *Aeromonas veronii* strain JCM 7375. All four strains are grouped together with a bootstrap value of 100%. The branching within the *Aeromonas* clade indicates varying degrees of divergence between the strains, with the *Aeromonas veronii* strain JCM 7375 being distantly related to the other members of the same genus. Silva *et al*. (2017) studied the genetic diversity of *Aeromonas* spp. isolated from the environment and also observed clade formation between the *Aeromonas caviae*, *Aeromonas hydrophila* and *Aeromonas veronii* species.

Stenotrophomonas koreensis strain TR6-01 is seen to branch off with 100% bootstrap support, forming an independent branch outside of the *Aeromonas* clade. This position indicates that *Stenotrophomonas koreensis* diverged from the *Aeromonas* group. The solitary placement of *Stenotrophomonas koreensis* from the other taxa indicates the absence of a common ancestor.

Brevundimonas diminuta strain NBRC 12697 and *Bacillus subtilis* strain IAM 12118 were grouped with 100% certainty, which indicates a common ancestor between the two species and the formation of a monophyletic clade. However, their placement indicates that they are more distantly related to the other groups, illustrating a separate lineage.

Providencia vermicola strain OP1 and *Providencia rettgeri* strain DSM 4542 form a distinct clade with 100% bootstrap support, which indicates their close evolutionary relationship and, as a result of the clade placement within the tree, the likely divergence from the remaining clades. Yuan *et al*. (2020) constructed a maximum likelihood phylogenetic tree with species of the *Providencia* genus obtained from soil, sewage, and freshwater environments and found that the relatedness of *Providencia rettgeri* and *Providencia vermicola* grouped together, therefore indicating the genetic relatedness between these species.

The different species detected at the different substrates indicate that bacterial attachment can be dependent on the chemical and surface properties of the substrates (Talagrand-Reboul *et al*., 2020). The *Aeromonas* spp. isolated originated from the HDPS and LDPE biofilms, indicating that a somewhat similar bacterial composition was observed. Low-density polyethylene has a lower density, and a softer and more hydrophobic surface compared to HDPE (Puglisi *et al*., 2019). Puglisi *et al.* (2019) studied the microbial colonization of polyethylene and found that distinct bacteria communities formed on the different polyethylene sources, resulting in different biofilm compositions. Figure 4.14 illustrated that *Aeromonas* spp. was found within the HDPS, HDPE and LDPE biofilms, and therefore the substrates act as possible vectors for the transportation and dissemination of ARG. *Stenotrophomonas koreensis* was only found in the nylon biofilm, possibly indicating that the growth of *Stenotrophomonas koreensis* is plastic specific (Deng *et al*., 2022). *Brevundimonas diminuta* is seen to be present in two different microcosms, namely wood and HDPS while *Bacillus subtilis* was only present in the wood microcosm. *Providencia* spp. was found within the HDPE and wood biofilm. Ultimately, wastewater was used for all of the microplastics, leading to a somewhat similar bacterial distribution between the different substrates.

5.6. Biofilm composition

5.6.1 Bacterial diversity

Microbiome diversity is defined as the abundance, composition, evenness, and richness of certain taxa as well as the interactions between the taxa (Nasir *et al*., 2023). Therefore, these parameters play an important role in the processes responsible for ecosystem functioning and stability (Nasir *et al*., 2023). According to Kim *et al.* (2017), the bacterial communities' features in a certain niche are characterised by the number of species present and their numerical composition, their bacterial diversity. Therefore, a variety of bioinformatics tools have been developed to determine the bacterial diversity of a sample. Shannon–Weaver and Simpson diversity indices are commonly used due to their Operational taxonomic unit (OTU) (Kim *et al*., 2017). Operational taxonomic units exist based on sequence data and are defined as different

levels of resolution namely, phylum, class, order, family, genus and species. Rarefaction can therefore be used to measure bacterial richness, whereas an abundance-based coverage estimator (ACE) and Chao1 are used to estimate the richness of a microbial community (Kim *et al*., 2017).

Kim *et al*. (2017) defined species richness as the number of different kinds of organisms present in a specific community compared to the relative abundance, which is seen as the uniformity of the population size of each species. The Shannon – Weaver Index places more importance on species richness and predicting the average degree of uncertainty, whereas the Simpson Index focuses more on the evenness of the species diversity and dominance along with reflecting the probability of two individual organisms belonging to the same species (Kim *et al*., 2017).

Abundance based-coverage estimator evaluates the total richness of a community of a sample, Chao1 and ACE had been developed to estimate the richness by calculating the expected OTU based on the observed OTUs (Kim *et al*., 2017). Chao1 is based on the concept that rare species infer the most information about the number of missing species due to more importance placed upon the lower species abundance and can therefore be useful for data that are skewed towards the lower species abundance (Kim *et al*., 2017).

Community profiling based on genus level indicated that river water had the highest species diversity and richness even though the dominance was lower (Figure 4.15 a-c). High-density polyethylene had a raised diversity and richness, but the species dominance was low, nylon presented low species richness but a high species dominance. The wastewater had a raised species diversity and richness but a lower species dominance, resulting in the abundance of different species without certain species being dominant, whilst nylon showed a lower species diversity and richness but a higher species dominance. The average DNA extraction of the substrates presented a higher concentration and quality at the wood substrate compared to HDPE which had the least DNA extracted (Figure 4.7). The average DNA extraction of the environmental samples indicated that the wastewater sample had a higher DNA concentration and quality compared to the river water sample (Figure 4.7). Zhang *et al* (2020) found that PE and PP plastic fragments surfaces were enriched with a variety of microbial communities compared to the surrounding aquatic environments. The community diversity estimators (Shannon and Simpson) also indicated that the bacterial community diversity of the microplastics was higher than that of the water samples; the same trend is seen in the findings of Zhang *et al*. (2020). Thus, the microbial biofilms of microplastics differ in composition from the surrounding water as indicated by the Shannon diversity, Simpson richness and evenness indexes.

β-diversity is defined as the variation in identities of species among sites, which makes assemblages of species similar to one another (Hardersen & Porta, 2023). The Bray Curtis (dis)-similarity is one of the most frequently used statistics for calculating β-diversity on abundance data, especially multivariant community data while the Jaccard similarity index is used to determine the taxonomic diversity in a biofilm (Bassiouni *et al*., 2015; Hardersen & Porta, 2023). The Jaccard similarity index is used for the characterisation of co-occurrence relations, and in doing so the similarity between communities (Koeneman & Cavanaugh, 2022; Ramette, A. 2007).

Figure 4.16 demonstrates the bacterial composition between the various substrates. In the nMDS plot using the Jaccard similarity index, the points grouped closely together indicate a greater community relatedness compared to the community dissimilarity observed at points further apart (Ramette, A. 2007). Nylon and HDPS are plotted close together, indicating that they host similar bacterial communities, which can suggest that certain bacteria colonize synthetic polymers (Ramette, A. 2007). Wastewater effluent samples are clustered with HDPE and LDPE, indicating the similarity between the bacterial communities while the positive control is plotted far away from the remaining substrates, indicating the distinct bacterial community found within the microcosm. The wastewater effluent and the river water were clustered together as well compared to the distinct bacterial community observed in some of the wood samples. The Jaccard similarity index suggests a better clustering of samples compared to the water sources. This correlates with the microplastic BCC greater compared to the surrounding environment, as seen by the species diversity, richness, and evenness.

Although all substrates were treated similarly, it should be noted that differences in community profiles could be induced by the considerable difference in the cell numbers adhering to the substrate shape and the adherence strength of the biofilm to the respective substrate surface as well as the substrate type (Kirstein *et al*., 2019). Biofilm communities are characterised by a diversity of organisms with varying metabolic capabilities and physiological traits. This diversity fosters both competition and opportunities for cooperation within the community (Kirstein *et al*., 2019). Hence, some of the observed changes in community composition might be related to organisms playing a specific role in interspecies interactions within the plastisphere (Kirstein *et al*., 2019; Schlundt *et al*., 2020).

5.6.2 Bacterial composition and profiling

Next-generation sequencing served as an identification tool for investigating the microbial community composition surrounding microplastics. The current study established and identified 14 different phyla, 20 classes, 19 orders, 16 families, 16 genera and 13 different

species within the microbial community. In this study, it is important to note that data analysis at the genus level demonstrated that many sequences were not classified at that level, and the lowest taxonomic classification was attained at the family level or, in some cases, even at the order level. In this study, certain bacterial groups were dominant in the biofilms surrounding the various substrates.

5.6.2.1. Taxonomic level: Phylum

The bacterial composition was dominated by the 5 most dominant bacterial phyla, namely, Proteobacteria (mean relative abundance = 37.77%), Planctomycetota (17.69%), Cyanobacteria (14.40%), Patescibacteria (10.13%) and finally Bacteroidota (7.69%) and these phyla accounted for approximately 87% of the total sequences (Figure 4.17). Thus, members of the phyla Proteobacteria and Planctomycetota were continuously identified as the dominant groups at the majority of the sample substrates, excluding nylon 1, positive control 2 and 4, river water 1 and 2 and wastewater 1. Oberbeckmann *et al*. (2014) and He *et al.* (2023) found that Proteobacteria was the most dominant microbial group on microplastics in aquatic environments globally and are considered early biofilm colonisers. Miao *et al.* (2019) found that the substrate type affected the composition and structure of biofilms, Proteobacteria was the dominant phylum collected in the biofilm samples, but Planctomycetota aided in variation with a -20 times higher abundance on the microplastics compared to the natural substrates, which supports these studies findings. Nylon 1 was dominated by the Bacteroidota phylum (59.5%), whilst positive control 1 and positive control 4 were dominated by Cyanobacteria (100%).

The wood substrates were dominated by Proteobacteria and Planctomycetota compared to Patescibacteria in the river water 1 (68.7%), river water 3 (67.0%) and wastewater 1 (64.5%) samples. Wood served as the non-plastic control (Figure 4.17). The substrates were incubated in the same water environment, therefore the bacteria in their biofilms are somewhat similar at the phylum level and revealed that the diversity of micro-organisms in biofilms is mainly determined by the primitive bacteria of the environment (He *et al*., 2023).

There were phyla detected with a mean relative abundance below 5%, namely, Campylobacterota (4.34%), Desulfobacterota (3.09%), and Verrucomicrobiota (2.94%). Other phyla with a mean relative abundance <1% included Nitrospirota, Actinobacteriota, Chloroflexi, Gemmatimonadota, Acidobacteriota and Firmicutes, as seen in Figure 4.17. Gao *et al* (2021), found similar results from water obtained at the WWTP, as obtained from this study.

According to Ting *et al*. (2021) and Bocci *et al*. (2024), common microbial communities within freshwater systems include members of Proteobacteria, Bacteroidetes, Actinobacteria,

Cyanobacteria, Verrucomicrobiae and Planctomycetes phyla. They have major roles in various biochemical cycles, such as nitrogen fixation, carbon recycling, and organic compound degradation within the aquatic ecosystem (Ting *et al*., 2021). Ting *et al*. (2021) determined the prokaryotic communities in urban water systems consisted of Proteobacteria, Bacteroidetes, Actinobacteria, Cyanobacteria and Firmicutes, which corresponds to the dominant communities detected in the current study. This suggests that WWTPs harbour environmental bacteria as well as pathogens, possibly from untreated wastewater or polluted water, into the natural water system.

Proteobacteria is seen as one of the largest divisions within the prokaryotes and accounts for most Gram-negative bacteria within aquatic environments (Gupta, 2000). This phylum consists of a complex assemblage of phenotypic and physiological attributes including phototrophs, heterotrophs, and chemolithotrophs and includes a large number of known pathogens such as coliforms and food pathogens (Gupta, 2000; Ting *et al*., 2021). The Proteobacteria phylum composition is derived from the 16S rRNA oligonucleotide catalogues, phylogenetic analysis and the formation of a distinct clade within phylogenetic trees based on the 16S rRNA/rDNA sequences, rRNA cistron similarities and along with the rRNA-DNA hybridisation results (Gupta, R. S. 2000). Zhang *et al* (2020) results presented that the phylum, Proteobacteria had the highest abundance (34.8 – 92.2%) detected from microplastic samples obtained from freshwater systems which correlate with the current studies result.

Initially classified as eukaryotes, members of the phylum Planctomycetota were classified as bacteria and are found in aquatic environments (Wurzbacher *et al*., 2024). Planctomycetota lacks peptidoglycan, form compartmentalised cells while possessing nucleus-like structures, and are capable of endocytosis-like mechanisms, whereby the latter is a hallmark of eukaryotes (Wurzbacher *et al*., 2024). Planctomycetota has a big impact on global biogeochemistry due to the ability to break down detritus particles in the aquatic environment (Wiegand, *et al*., 2018). Godinho *et al*. (2024) studied the phylum Planctomycetota and found that resistance towards A10, VA30, T5, K30, and C30 is present and detected in hotspots for ARB dissemination, such as in WWTP. This is in line with the current study as observed by the pathogenic potential of the isolated bacteria.

Cyanobacteria are commonly found in surface- and groundwater but can also grow and proliferate in dark conditions (Nasir *et al*., 2023). They form part of the microbial biofilm community, and their growth can be influenced by temperature and nutrients The EPS produced by Cyanobacteria has adhesive properties that contribute towards the aggregation of microbes on microplastics and can influence the buoyancy of these particles resulting in the movement within the water column (Gopalakrishnan & Kashian, 2023; Yokota *et al*., 2017). In

environments subjected to intense anthropogenic activities, such as WWTP, dominant cyanobacteria can occur with biological toxins, chemical pollutants, and pathogenic microbes (Metcalf & Codd, 2020). Cyanobacterial cells proliferate in municipal WWTP, which flourish in steady, nutrient-rich environments and are favoured by the extended water residence times, such as in the case of the positive control microcosm (Ahmad, 2021; Qiang *et al*., 2021). In some instances, Cyanobacteria is used in WWTP with the purpose of aiding in bioremediation and metabolising nutrients along with acquiring natural resistance and selectivity against environmental pollutants due to their presence in polluted systems (Ahmad, 2021). The efficacy of cyanobacteria within wastewater treatment relies on the strain's ability to propagate under environmental conditions and the efficiency of discharging the contaminates (Ahmad, 2021). Urbanised water systems or anthropogenic activities with close proximity to water systems lead to higher concentrations of nutrients available, resulting in an increase in cyanobacteria growth and replication (Ahmad, 2021; Ting *et al*., 2021). Cyanobacteria was the most abundant in the positive control microcosm, thriving in the stagnant water and, in doing so, dominating the microbial community. The continuous detection of Cyanobacteria at different microcosms, but in lower abundance, indicates that the source of the cyanobacteria could have been from the WWTP. In this study, Cyanobacteria were detected, but results did not discriminate if these were symbionts, free-living, or toxic cyanobacteria. The Beta diversity also confirms the identical species composition of the positive control microcosm Qiang *et al*. (2021) suggested that to limit the amount of Cyanobacteria growth within a freshwater microcosm, the microcosm should be placed in a fridge to decrease the possibility of cyanobacteria completely colonising the microcosm, as it did in the case of positive control 2 and 4 sample.

The river water and wastewater 1 samples were dominated by the Patescibacteria phylum. Patescibacteria or Candidate Phyla Radiation (CPR), which are found in a variety of environments, including activated sludge from WWTP, groundwater and freshwater; they exhibit parasitic abilities with limited metabolic potential and fermentation-based metabolism (Fujii *et al*., 2022; Hu *et al*., 2024). The term Candidate Phyla Radiation (CPR) was originally proposed for these organisms and was defined as a superphylum radiation that contains more than 74 phyla (Hu *et al*., 2024). The absence of the rpL9 and rpL1 ribosomal protein in the *Microgenomatia* and *Saccharimonadia classes* is an indication of the compositional and structural peculiarities within the patescibacterial ribosomes (Hu *et al*., 2024). Hu *et al*. (2024) studied the prevalence and identification of Patescibacteria in sewage. The study revealed that due to the epiparasitic nature of Patescibacteria, the possible hosts could be the species of the following phyla: Acidobacteriota, Bacteroidota, Campylobacterota, Chloroflexi, Actinobacteriota, Firmicutes, Nitrospirota, Proteobacteria, Planctomycetota and

Verrucomicrobiota (Hu *et al*., 2024). Hu *et al*. (2024) also found that the relative abundance of Patescibacteria was several magnitudes higher than originally thought by Dueholm *et al* (2022). The current study revealed that the presence of Patescibacteria might be attributed to the prevalence of the above-mentioned hosts.

5.6.2.2. Taxonomic level: Class

Alphaproteobacteria dominated the bacterial Class composition (mean relative abundance – 31.64%), Phycisphaerae (14.83%) and Cyanobacteriia (12.99%), followed by Bacteroidia (7.7%), Gracilibacteria (6.41%), Gammaproteobacteria (6.13%), Campylobacteria (4.35%), Unassigned (3.09%), Planctomycetes (2.87%), Saccharimandia (2.60%), Verrucomicrobiae (2.27%), Vampirivibrionia (1.41%) and Parcubacteria (1.08%). Classes with a mean relative abundance <1% included, Chlamydiae, Nitrospiria, Actinobacteria, Anaerolineae, Gemmatimonadetes, Vicinamibacteria and Clostridia as seen in Figure 4.18.

Alphaproteobacteria is the most prevalent class identified in all of the HDPE, LDPE and HDPS samples and ranged between 30.1% and 69.5%. Nylon 1 had Bacteroida as the dominant class (59.5%), while positive control 2 and positive control 4 were dominated by Cyanobacteria (100%). Gracilibacteria dominated the river water 1 (48.1%), river water 3 (41.3%) and wastewater 1 (45.3%) samples, compared to the wastewater 2 (29.2%) and wood 1 (33.7%) samples that were dominated by the Phycisphaerae class (Figure 4.18).

Proteobacteria is divided into 5 subclasses, Alpha-, Beta-, Gamma-, Delta-, and Epsilonproteobacteria (Gupta, R. S. 2000). The class Alphaproteobacteria is seen as one of the largest groups within the Proteobacteria phylum, which includes phototrophs, chemolithotrophs, chemoorganotrophs, and aerobic photoheterotrophs, which are abundant in aquatic and terrestrial environments along with frequently adopting an intracellular lifestyle as plants mutualists or animal pathogens (Gupta & Mok, 2007, Williams *et al*., 2007). Alphaproteobacteria is divided into seven orders, namely, Caulobacterales, Rhizobiales, Rhodobacteriales, Rhodospirillales, Rickettsiales, Sphingomonadales and Pharvularculales (Gupta & Mok, 2007). These subgroups are distinguished based on their branching in phylogenetic trees as well as protein specificity and indels (Gupta & Mok, 2007, Brilli *et al*., 2010). Alphaproteobacteria are seen as the primary colonisers within biofilms and are often considered the pioneers of biofilm formation (Nasir *et al*., 2023). Tamminen *et al*. (2022) investigated the biofilm communities in river water systems influenced by WWTP effluent, where they found that within the streams biofilms, Proteobacteria and Planctomycetota were detected upstream as well as downstream of a WWTP discharge point, resulting in an insignificant influence caused by the WWTP effluent. Tamminen *et al*. (2022) findings aligned

with the current study in regard to the biofilm composition around the microplastics and within wastewater 2 and -3 samples.

Planctomycetota is divided into two classes, namely, Phycisphaerae and Planctomycetia and comprises 7 orders, 10 families and 60 genera (Godinho *et al*., 2024; Lenferink *et al*., 2024). The current study found that both classes are present within the biofilms. Phycisphaerae comprises 9 species and is divided into 3 orders, Phycisphaerales, Sedimentsphaerales and Tepidispaerales, compared to Planctomycetes which consists of 99 species and 4 orders; Gemmatales, Isophaerales, Pirellulales and Planctomycetes (Godinho *et al*., 2024). In the current study, wastewater 2 and wood 1 were dominated by the class Phycisphaerae.

The class Cyanobacteriia was the third abundant class found within the microbial communities on the different substrates. Gracilibacteria, Parcubacteria and Saccharimonadia were detected in the river water and wastewater 1 sample. These classes are predominately found within activated sludge and groundwater while belonging to the Patescibacteria phylum (Hu *et al*., 2024). According to Hu *et al*. (2024), 3 genera of *Saccharimonadia* have been co-isolated with bacterial hosts from WWTPs, and all have been shown to have an epiparasitic lifestyle with the host of the phylum Actinomycetota.

5.6.2.3. Taxonomic level: Order

The order of the microbial composition was dominated by Caulobacterales (mean relative abundance – 14.50%), Chloroplast (13.42%), Unassigned (10.30%), Phycisphaerales (8.92%), Chitinophagales (8.16%) and Tepidisphaerales (7.25%), which is then followed by Kiloniellales (5.49%), Campylobacterales (4.71%), Ferrovibrionales (4.53%), Pseudomonadales (3.83%), Rhizobiales (3.72%), Saccharimonadales (2.83%), Burkholderiales (2.78%), Acetobacterales (2.23%), Sphingomonadales (1.79%), Planctomycetales (1.72%), Obscuribacterales (1.55%), Verrucomicrobiales (1.14%) and lastly Chthoniobacterales (1.00%) as seen in Figure 4.19.

The HDPE and LDPE samples demonstrated that the Caulobacterales order was most prevalent and ranged from 18.1% and 37.4% whilst at positive control 2 and positive control 4, Chloroplast was dominant (100%), HDPS 2 (24.7%) and wastewater 2 (31.4%) samples indicated that Phycisphaerales was the most abundant order detected. The nylon samples had Chitinophages as the dominant order (23.8% - 60.6%), Tepidisphaerales was the most abundant order at the wastewater 3 (19.9%), wood 1 (26.0 %) and wood 3 (31.7%) samples (Figure 4.19).

The Order Caulobacterales is defined as chemoorganotrophic bacteria that are commonly found in aquatic aerobic environments and are distinguished by their ability to form stalked cells and an unusual cell division cycle (Gupta & Mok, 2007). According to Lee *et al*. (2005), Caulobacterales belongs to the Alphaproteobacteria class and the Proteobacteria phylum and can be subdivided into three families, namely, Caulobacteraceae, Hyphomonadaceae and 'Rhodobacteraceae'.

The order Phycisphaerales belongs to the Planctomycetota phylum and can be found in a multitude of environments, such as in soil, wastewater and aquatic environments (Lenferink *et al*., 2023). Members of this order are known for their adaptability to aquatic ecosystems and their complex carbon degradation processes (Lenferink *et al*., 2023).

The order Chloroplast belonging to the class Cyanobacteriia was detected on the positive control 2 and -4 samples. Chloroplasts are metabolically active, semi-autonomous organelles found in plants, algae and cyanobacteria (Dobrogojski *et al*., 2020). The presence of the Chloroplast order can be a result of plant or algal material, misclassification of taxonomy and the improper amplification of 16S rRNA genes (Beckers *et al*., 2016).

5.6.2.4. Taxonomic level: Family

Hyphomonadaceae was found to be the most abundant family within the microbial community surrounding the substrates, with a mean relative abundance of 14.71%, excluding the Unassigned group (26.22%) due to incomplete classification of the bacterial orders, caused by the limited data collected to correctly identify the unassigned group. Thereafter, Phycisphaeraceae (9.28%), WD2101 soil group (7.60%), Fodinicurvataceae (5.97%), Chitinophagaceae (5.71%), Arcobacteraceae (5.09%), Ferrovibrionaceae (4.76%), Moraxellaceae (3.98%), Rhodocyclaceae (2.88%), Saprospiraceae (2.73%), Acetobacteraceae (2.42%), Xanthobacteraceae (2.30%), Sphingomonadaceae (1.89%), Rubinisphaeraceae (1.64%), Obscuribacteraceae (1.62%) and lastly Verrucomicrobiaceae (1.18%) as seen in Figure 4.20.

This study found that Hyphomonadaceae to be the dominant family at HDPE (18.2% - 39.9%), LDPE (22.7% - 26.1%), nylon 2 (19.5%), nylon 3 (22.4%), HDPS 1 (28.7%), and HDPS 1b (33.6%) samples are. HDPS 2 (25.1%) and wastewater 2 (31.5%) samples had Phycisphaeraceae as the dominant family. Wastewater 3 (20.6%), wood 1 (26.9%) and wood 3 (33.8%) indicated that the WD2101 Soil group the dominant family is. Fondinicurvataceae is found to be the dominant family in the wood 2 sample (67.7%). Nylon 1 sample had Chitinophagaceae as the dominant family (61.2%), while Moraxellaceae was dominant at HDPS 3 (17.7%). Positive controls 2 and 4 could not be identified due to being unassigned

family level (100%), river water 1, river water 3 and wastewater 1 ranged from 50.9% to 55.2% unassigned. Arcobacteraceae was detected in river water 1 and 3 and wastewater 1 and ranged between 32.5% and 37.5% (Figure 4.20).

He *et al* (2023) found that on HDPE, Caulobacteraceae, the most dominant family is, while Sphingobacteriaceae and Sphingomonadaceae were also present. Sphingomonas were detected in HDPE biofilms due to the hydrophobic materials and less on hydrophilic materials, the bacterial content is therefore 10 times higher on the microplastic than the surrounding environment (He *et al*., 2023). The current study differed from He *et al* (2023) finding in the sense that the dominant families detected on the HDPE substrates were Hyphomonadaceae. The family Hyphomonadaceae belongs to the order Rhodobacteriales class Alphaproteobacteria and Proteobacteria phylum; members of this family can be found in aquatic environments (Lee *et al*., 2005, Ajvazi *et al*., 2022; Asem *et al*., 2020).

5.6.2.5. Taxonomic level: Genus

The Unassigned genus mean relative abundance of 42.23% illustrates that more than 40% of the microbial community could not be classified due to incomplete databases and previously un-sequenced isolates. Unassigned genera can also be attributed to the lack of sufficient data, conflicting results with conflicting methods, poor quality sequencing data due to sample mishandling, chimeric sequences, partial genome sequencing, non-culturable microbes, mistagging or unsuccessful tagging of barcodes to the intermediate PCR products during library construction (Chen *et al*., 2021).

SWB02 is thereafter the dominant genus (15.25%), followed by *SM1A02* (9.50%), *Arcobacter* (5.20%), *Ferrovibrio* (4.96%), *Edaphobaculum* (4.84%), *Acinetobacte*r (4.09%), *Lewinella* (2.79%), *Methyloversatilis* (2.55%), *Roseomonas* (2.46%), *Sphingomona*s (1.92%), *Pseudolabrys* (1.69%), *SH-PL14* (1.24%) and *Verrucomicrobium* (1.20%) as seen in Figure 4.21.

The prevalent genus detected at HDPE, LDPE, nylon 2, nylon 3, HDPS 1 and HDPS1b were *SWB02,* and the mean abundance ranged between 19.1% and 41.9%. *SM1A02* was the dominant genus at nylon 1 (15.0%), HDPS 2 (25.2%) and wastewater 2 (31.5%). *Ferrovibrio* is the most dominant genus found on HDPS 3 (26.1%). Positive control 2 and -4, both with a relative abundance of 100%, river water 1, and -3, wastewater 1 and -3 and all the wood biofilm samples could not be identified due to a shortage in isolated genes in bacterial gene libraries as well as the possibility of undiscovered bacterial species (Figure 4.21).

River water 1 and -3, along with wastewater 1, illustrated that *Acrobacter* was thereafter the dominant genus (33.3% - 38.3%). *SWB02* was detected at wastewater 2 and -3 as well as wood 2 samples and ranged from 11.0% to 19.8% of the microbial community composition. *Roseomonas* sp. was detected at wood 2 and *Sphingomonas* at the wood 3 biofilm sample. The results obtained demonstrated various bacterial community compositions within the surrounding biofilms of the various substrates and water sources. Thereby illustrating the diversity and abundance within biofilms.

Sheridan *et al*. (2022) tested the microbial composition of LDPE leachate within freshwater systems; 20 lakes were subjected to 16S amplicon sequencing, with *Acinetobacter* dominating the LDPE community composition. The current study illustrated that *Acinetobacter* was detected surrounding the LDPE substrates but in a decreased percentage. Therefore, Sheridan *et al*. (2022) confirmed the results to some extent.

5.6.2.6. Taxonomic level: Species

The Unassigned specie's mean relative abundance of 43.61% indicates that only 56.39% of the bacterial community composition could be identified up to the species level. *SWB02* sp. is the second most abundant species (15.62%), followed by *SM1A02* sp. (9.71%), *Arcobacter cryaerophilus* (5.27%), *Edaphobaculum* sp. (4.86%), *Ferrovibrio* sp. (4.48%), *Acinetobacter_lwoffii* (3.10%), *Lewinella*_sp. (2.87%), *Roseomonas*_sp. (2.62%), *Methyloversatilis*_sp. (2.61%), *Pseudolabrys*_sp. (1.8%), *SH-PL14*_sp. (1.26%), *Verrucomicrobium_spinosum* (1.24%) and lastly, *Acinetobacter_guillouiae* (0.09%), as seen in Figure 4.22.

The results found in this study demonstrate that *SWB02* sp. is the most abundant species found in the HDPE, LDPE, nylon 2, nylon 3, HDPS 1, HDPS 1b, and HDPS 2; the relative abundance ranged from 19.6% to 44.2% in these biofilms. The *SM1A02* sp. dominated the wastewater 2 biofilm sample while *Ferrovibrio* sp. was the dominant species in the HDPS 3 biofilm. Nylon 1 biofilm was dominated by Edaphobaculum sp., river water 1 and -3, and wastewater 1 samples contained *Arcobacter cryaerophilus* as the identifiable prevalent species (ranging between 33.7% and 38.3%) (Figure 4.22).

Positive control 2 and -4, both with a relative abundance of 100%, the river water and wastewater 1 and -3 samples, along with the wood biofilm sample, consisted of unassigned bacterial species. The least diversity was observed in the river water and wood microbial composition samples compared to HDPE and LDPE, which demonstrated the most diversity. Yang *et al.* (2020) and Oberbeckmann *et al*. (2018) are corroborated by the current study's

results, which illustrate the difference in microbial communities on different substrates as well as different water sources.

5.6.2.6.1 *SWB02*

The SWB02 species belongs to the Hyphomonadaceae family. They can utilise volatile fatty acids in aerobic conditions and enhance the electron transfer between microbes and substances, but they also form part of carbon cycling and are associated with plant-interacting microorganisms. (Tang, *et al*., 2023; Zhang *et al*., 2022). *SWB02* was the dominant species detected at the HDPE, LDPE, nylon2, nylon3, HDPS1, HDPS1b, and HDPS2 particles.

5.6.2.6.2. *SM1A02*

SM1A02 species originates from the WWTP and belongs to the Phycisphaeraceae family, Planctomycetes (Vico *et al*., 2021) The *SM1A02* species plays an important role in carbon and nitrogen cycling, they perform anaerobic ammonium oxidation, thereby decreasing the nitrogen concentration within water sources, especially in WWTP but can also be present in brackish water (Lenferink *et al*., 2023; Wu *et al*., 2023). Lenferink *et al* (2023) found that SM1A02 can adapt to extreme environments and may depend on amino acids for its survival within the aquatic environment.

5.6.2.6.3. *Ferrovibrio*

Ferrovibrio genus was first proposed by Sorokina *et al* (2012) and currently consists of three species, *Ferrovibrio denitrificans*, *Ferrovibrio soli,* and *Ferrovibrio xuzhouensis* and belongs to the Rhodospirillaceae family and the Proteobacteria phylum (Dahal & Kim, 2018; Ryu *et al*., 2020). *Ferrovibrio* species are Gram-negative, aerobic or facultatively anaerobic, motile, catalase- and oxidase positive, along with K30 and T5 resistance (Ryu *et al*., 2020).

5.6.2.6.4. *Edaphobaculum* sp.

Edaphobaculum sp. can be described as Gram-negative, aerobic, rod-shaped and non-motile bacteria belonging to the Chitinophagaceae family and the Bacteroidetes phylum (Cao *et al*., 2017). *Edaphobaculum* sp. produces lipase but presents sensitivity towards VA30 and resistance towards T5 (Cao *et al*., 2017).

5.6.2.6.5. *Arcobacter cryaerophilus*

The genus *Arcobacter* belongs to the Proteobacteria family and consists of eight species namely *Arcobacter cryaerophilus, Arcobacter butzleri, Arcobacter skirrowii, Arcobacter cibarius, Arcobacter thereius, Arcobacter halophilus, Arcobacter mytili, and Arcobacter*

nitrofigilis (Houf *et al.,* 2000). *Arcobacter cryaerophilus, Arcobacter skirrowii, Arcobacter cibarius and Arcobacter thereius* is found in livestock, animal-based food, and water and is associated with human diarrheal illness and bacteraemia (Houf *et al.,* 2000; Debruyne *et al*., 2010). *Arcobacter cryaerophilus* was seen to be resistant towards C30, T5 and VA30 (Fera *et al*., 2003).

5.7. Summary of Discussion

High density polystyrene, HDPE, LDPE, nylon, and wood particles were incubated for 28 days in a simulated freshwater environment. Throughout the 28 days, sampling took place within each microcosm; 10 substrate particles were collected and plated onto R2A agar, from which plating was done to obtain pure bacterial colonies. 877 pure colonies were collected and underwent extracellular enzymatic tests to determine the virulence associated with the isolates. A value was assigned for each positive virulence test to gain a virulence score for each isolate. Bacterial isolates with a virulence score above 3.5 were selected for further characterisation, thereby eliminating the lesser virulent.

51 isolates underwent Gram staining and the Kirby Bauer disc diffusion method. It was found the majority of the isolates were Gram-negative (72.5%). The isolates exhibited resistance to various antibiotics, including T5, A10, and C30. Based on the varying degrees of resistance towards the different types of antibiotics, it can be seen that K30 and C30 proved to be the most effective antimicrobial agent used against the isolated bacteria, while A10 proved to be the least effective antimicrobial agent used against the isolated bacteria, followed by T5.

The PPI was constructed to determine which of the bacterial isolates could be potentially pathogenic. Out of the 51 bacterial isolates tested for antibiotic resistance, 16 isolates presented multidrug resistance which can have possible health implications.

Those sixteen isolates were used downstream for Sanger sequencing. The most prevalent species included *Aeromonas* spp., *Brevundimonas* sp. and *Providencia* sp. From the pure isolated cultures and the biofilms surrounding the microplastics, potentially pathogenic bacteria are present and can be attributed to the EPS of biofilms. From the 16 potentially pathogenic bacteria, HDPS and wood substrate had the most potentially pathogenic bacteria present within their biofilms, while Nylon, HDPE and LDPE had the least amount of potentially pathogenic bacteria present.

Next-generation sequencing led to the identification of the dominant phylum, class, order, family, genus, and species. The most dominant phylum was Proteobacteria, the class was Alphaproteobacteria, the order was Caulobacterales, the Family was Hyphomonadaceae, the genus was *SWB02,* and the species was *SWB02 sp*. The various substrates exhibited distinct bacterial communities, with the increased possibility of genetic exchange resulting in increased antibiotic-resistance genes within the environment. The various species detected originated from wastewater and aquatic environments.

Chapter 6 : Conclusion

Water is essential for human survival and the long-term development of ecosystems. Global pollution, urbanisation and industrialisation resulted in an increased demand for freshwater sources (Phungela *et al*., 2022). Large amounts of plastic debris are continuously released into the environment, whether directly or indirectly (Miao *et al*., 2019). The decline in municipal WWTP and infrastructure is seen as one of the most significant contributors to pollution in water sources; the effluent generated by industrial and home use is seen as the second most common source of chemical and microbial pollution of SA's surface water sources (Phungela *et al*., 2022). The decreased WWTP capacity and infrastructure results in the introduction of ARB and microplastics in the surface water (Verster *et al*., 2017). Microplastics are identified as an emerging environmental threat to aquatic ecosystems due to their negative impacts on a range of aquatic organisms, from phytoplankton to fish, as well as their ability to act as vectors for potentially pathogenic microbial communities (Miao *et al*., 2019; Oberbeckmann *et al*., 2018). Research on detecting microplastics in South African freshwater sources has increased significantly in the last 8 years (Verster *et al*., 2017; Pereao *et al*., 2020; Naidoo *et al*., 2019; Weideman *et al*., 2020; Nel *et al*., 2018; Dahms *et al*., 2020; Ramaremisa *et al*., 2022).

This study aimed to characterise and identify the bacterial composition surrounding microplastics by stimulating freshwater environments. This sheds light on the microbe-microplastic interaction when microplastics and wood substrates are introduced into a microcosm for 28 days. The study illustrates that microbial colonisation on various substrates occurred within 72 hours of contact, forming biofilms in nutrient-rich water (WWTP effluent) and illustrating the potential impact on the aquatic environment.

Following the microcosm experiment, another objective was to determine the characteristics of pure microbes through various extracellular enzymatic and antibiotic susceptibility tests and, in doing so, identify the antibiotic-resistant bacteria within the aquatic environment. Hereafter, the summaries of each objective are provided.

6.1. Objective 1: Conduct microcosm studies to investigate biofilm formation on various substrates using scanning electron microscopy.

Microcosms were used to simulate the freshwater environment to gain a better understanding of the microbe-microplastic interaction in freshwater environments. The most common

polymers within the environment are dominated by six substances, namely PS, PVC, PE, PP, PET and PUR (Pareao *et al*., 2020; Thakur *et al.*, 2023). In this study, four (4) different microplastics (HDPE, LDPE, HDPS and Nylon) and one (1) non-plastic control (wood) substrate were incubated in a mixture of river water and WWTP effluent for 28 days. The SEM images indicated that microbes attach more readily to uneven surfaces, such as pits and grooves of these polymers, than even surfaced ones. Surface degradation was observed between the pre-incubated and post-incubated microplastics. Surface degradation can affect the reactivity and potential toxicity of the microplastics (Pareao *et al*., 2020; Burrows *et al*., 2020).

The substrates that weren't incubated for the 28-day period illustrate a difference in surface properties compared to the incubated substrates. The study observed biofilm formation on both microplastics and wood substrates. Regarding substrate types, LDPE and HDPE were solid fragments and had smooth surfaces before incubation; post-incubation presented bacterial attachment within the grooves and crevices of the microplastics (Figures 4.1 and 4.2). Wood pre-incubation indicates an uneven surface whereby, after incubation, microbial cells are attached to the grooves, fissures and pits, which can be attributed towards the complex design of the particles.

The results indicated that the microplastics and wood substrates were niches for microbial attachment and the formation of communities through colonisation. The simulated environment was of no hindrance towards the bacterial attachment. In an environmental setting, the fate of microplastics within the aquatic environment is determined by the environment in which they find themselves; within moving water, the microplastics are transported throughout the water column and, in doing so, acquire different microbes and genes (Perveen *et al*., 2022). Still, within a simulated environment, microbe variation is limited due to the absence of external factors, such as rainfall, as seen in this study.

6.2 Objective 2: Characterization of pure bacterial isolates from biofilms surrounding microplastics.

Following the microcosm experiments and the visualisation of the biofilms, R2A agar was employed to aid in the procurement of pure bacterial colonies through plastic plating and streak plating. Pure bacterial colonies underwent various virulence tests through the use of differential media. The virulence testing led to the exclusion of the majority of the bacterial isolates by placing a numerical value on each positive enzymatic test.

The current results indicated that 49% of the isolated bacteria indicated a haemolytic capability, 32% tested positive for Proteinase, 27% for DNase and 38% for Lipase activity. The 877

bacterial isolates were lessened to 51 isolates. Thereby, only 6% of the bacterial isolates were used for further identification and characterisation, and of that 6%, 27.4% were Gram-positive, and 72.5% were Gram-negative. Gram-negative bacteria are responsible for several infections and present a decreased susceptibility to antibiotics (Evans *et al*., 2007), as proved by this study.

6.3 Objective 3: Determining the antibiotic resistance of the isolated bacteria.

Antibiotic-resistant bacteria need to be identified and classified within the environment to understand what influences antibiotic resistance. WWTP are seen as reservoirs for ARB due to the increased concentration of antibiotic-resistance substances and pathogenic bacteria, thereby releasing ARB and ARG into the receiving environment (Perveen *et al*., 2022; Geyer *et al*., 2017). Therefore, microplastics that are present in WWTPs, can act as vectors for pharmaceuticals and heavy metals and ultimately assist with the dissemination of ARG into the surrounding plastisphere. In SA, 80% of the WWTPs lack infrastructure and updated equipment to successfully remove microplastics and antibiotic-resistance elements (Perveen *et al*., 2022). Therefore, the amount of microplastics and pathogenic bacteria released into the freshwater environment is a cause of concern due to the rural areas' usage of these water sources (Naidoo *et al*., 2020).

After the isolated microbes were characterised, AST was done through the Kirby-Bauer disc diffusion method on the 51 bacterial isolates obtained from the virulence testing. Five different antibiotics were used with varying degrees of strength. The most common daily antibiotics, trimethoprim and amoxicillin, pose the most significant risk due to prolonged overexposure in the environment (Larsson, 2014), as observed in this study.

The microcosm study revealed that the isolated bacteria have varying degrees of antibiotic resistance. Through the isolation of ARB, one can assume that ARGs were present in the plastisphere and may have been transferred via HGT into the surrounding bacterial communities. The current results illustrated that A10, the least susceptible antibiotic, was compared to C30l, which was the most susceptible. The PPI indicated that sixteen isolates were awarded the highest pathogenic potential score due to resistance towards various antibiotics as well as exhibiting virulence factors.

6.4 Objective 4: Identification through Sanger sequencing

16 Bacterial isolates were chosen for Sanger sequencing after completing the extracellular enzymatic and antibiotic resistance tests. The chosen isolates presented a higher pathogenic potential than the remaining isolates. The sequenced isolates were subjected to identification through Sanger sequencing. Eight (8) different species were identified, and the sequences with high similarities (≥ 94%) were used for phylogenetic analysis. *Aeromonas hydrophila, Aeromonas caviae, Stenotrophomonas koreensis, Bacillus subtilis, Brevundimonas diminuta, Providencia vermicola, Aeromonas veronii and Providencia rettgeri* were identified.

A maximum likelihood phylogenetic tree was constructed to indicate the relationship between the various species, where a bootstrapping value indicated the amount of evolutionary certainty. A scale was also applied to the phylogenetic tree to indicate the amount of genetic change. Distinct clades with high bootstrap support (100%) were illustrated, an *Aeromonas* clade and a *Providencia* clade; *Stenotrophomonas koreensis* was seen to form an intermediate branch indicating a distant ancestor in common with the *Aeromonas* clade. A cluster consisting of the *Bacillus subtilis* and *Brevundimonas diminuta* indicates a more distantly related lineage.

6.5. Objective 5: Characterisation of the bacterial community composition on microplastics.

This study was performed to gain a better understanding of the bacterial community composition surrounding the microplastics. Following the completion of the microcosm experiment and SEM observations of the microbial community, the distribution and composition of the microbial assemblages on the different substrates and the two distinct water sources were investigated.

The diversity indices indicated that HDPE had the highest species richness and diversity compared to the remaining substrates but presented a lower dominance. The wood substrate measured the highest species dominance but had lower diversity and richness, indicating that the different substrates were colonised by other species and varying amounts of species. A comparison between the river water and wastewater samples indicated that river water had a higher species diversity and richness compared to the higher species dominance detected in the wastewater sample. The positive control microcosm presented a higher species dominance but a decreased species richness and diversity. Microbial community profiling observed in the microcosm studies and other research studies (Mayday *et al*., 2024) showed that microplastics were less rich and less diverse than in the surrounding water community.

The bacterial composition surrounding the various substrates were mainly dominated by the Proteobacteria and Plactomycetota phyla, as supported by Nasir *et al*. (2023) and Oberbeckmann *et al.* (2018). The distribution and composition of different dominant phyla were observed across the different substrates and source water samples (WWTP effluent and river water). The positive control samples showed that only one bacterial phylum was detected. The phyla distribution varied among the substrates and sample types. These differences could be attributed to the microbial colonisation process, the metabolic pathways involved and the plastic properties (Maday *et al.,* 2024)

The bacterial composition was then further investigated, revealing that Alphaproteobacteria, Phycisphaerae and Cyanobacteria were the dominant classes. According to (Nisar *et al*., 2023), Alphaproteobacteria are seen as the primary colonisers within biofilms and are often considered the pioneers of biofilm formation. In this study, Alphaproteobacteria (31.64%) was found to be the most dominant and is supported by Nasir *et al*. (2023) findings. It is important to note that the uniqueness and complexity of the microbial community led to a fraction of unassigned microbes.

South Africa is a drought-prone, water-poor region. Freshwater sources are limited, and the introduction of foreign materials and chemicals into freshwater sources results in the degradation of ecosystems and increased polluted water sources. This study demonstrated that microplastic serves as a substrate for microbial colonisation, possibly altering survival strategies and negatively affecting the ecosystem.

The collected data for the current study demonstrated that potentially pathogenic bacteria are present in biofilms surrounding various microplastics within freshwater systems in SA. By characterising the bacteria isolated from the substrate surrounding the microbial community, it can be concluded that the aim and objectives of this study were successfully achieved.

6.5. Recommendations

The findings of this research gave insight into the microplastic-micro-organism interactions in simulated freshwater environments through various techniques. However, more research is required to understand these interactions. The following recommendations are stated for future studies.

- Gain a better understanding of the interaction between microplastics and other contaminants and how the microplastics can function as vectors for chemicals, chemical properties along with polymer types should be studied to determine the type of chemicals that are released into the aquatic environment and how that affects the environment.
- Develop standardised methods and guidelines for the culturing of bacteria from different environments obtained from microplastics to create uniformity between different environments.
- Seasonal fluctuations should also be considered, for example, the dry and wet months and how they influence the microbial colonisation and distribution of microplastics by continuous sampling and monitoring throughout the annual period.
- The environment should be taken into account to construct a topographical map to illustrate the microplastic dispersal within the environment.
- Removal methods of microplastics from WWTP effluent should be implemented to decrease the amount of microplastics entering the aquatic environment.
- Continuous sampling and sequencing of biofilms obtained from different sources is recommended to aid in identifying and updating genetic libraries.
- Comparing the different polymer types and the bacterial colonisation across various freshwater environments.
- River sediments should be considered to act as repositories for microplastics and compared against various sediments from streams and rivers, aiding in the understanding of temporal distribution within the water column.

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Appendix A

Table A1: Antibiotic susceptibility tests inhibition size diameter (mm).

Isolate number	New number	VA30	A10	K30	C30	T5
131	1	17	8	24	24	0
147	2	17	8	20	19	0
158	3	8	0	21	27	15
170	4	15	8	21	21	0
198	5	23	11	23	26	0
199	6	17	10	21	25	0
232	7	21	17	31	28	26
249	8	15	0	20	18	0
261	9	15	8	25	19	0
264	10	16	0	16	19	0
285	11	16	8	18	26	0
342	12	16	11	17	34	0
358	13	8	0	15	20	14
359	14	8	0	16	21	8
364	15	8	8	17	21	12
365	16	0	8	15	9	0
374	17	8	8	22	11	10
381	18	17	0	23	20	0
420	19	18	16	20	27	0
433	20	0	12	23	14	16
496	21	19	33	29	30	0
507	22	18	40	32	35	0
508	23	0	0	13	12	0
509	24	17	12	19	25	0
510	25	19	11	30	26	25
511	26	9	44	33	35	18
555	27	0	0	13	9	0
564	28	17	35	30	28	0
645	29	0	13	11	15	0
666	30	19	22	31	26	26

682	31	16	30	23	30	34
695	32	8	36	31	40	0
696	33	8	16	12	21	0
709	34	17	8	19	27	0
722	35	18	0	20	27	0
725	36	0	0	13	20	8
729	37	15	11	16	16	0
730	38	8	8	17	22	15
731	39	8	7	14	19	13
733	40	9	0	15	22	15
747	41	8	8	22	24	20
765	42	0	10	23	11	8
767	43	8	0	21	27	17
774	44	10	0	11	17	8
779	45	0	22	20	25	17
816	46	0	0	10	15	0
818	47	10	0	21	28	20
821	48	10	0	21	30	20
822	49	10	0	21	28	17
824	50	15	0	19	12	0
875	51	15	9	19	21	0

Table A2: Pathogenic Potential Index

Isolate Number	Antibiotic Resistance (mm)					Virulence Characteristics				Pathogenic Score
	VA30	A10	K30	C30	T5	Haemolysis	Proteinase	DNase	Lipase	
1	0	1	0	0	1	1	1	1	1	6
2	0	1	0	0	1	0,5	1	1	1	5,5
3	1	1	0	0	0.5	0,5	1	1	1	6
4	0	1	0	0	1	1	1	1	1	6
5	0	1	0	0	1	1	1	1	1	6
6	0	1	0	0	1	1	1	1	1	6
7	0	0.5	0	0	0	0,5	1	1	1	4
8	0	1	0	0	1	1	1	1	1	6
9	0	1	0	0	1	1	1	1	1	6
10	0	1	0.5	0	1	1	1	1	1	6,5
11	0	1	0	0	1	0,5	1	1	1	5,5
12	0	1	0.5	0	1	0,5	1	1	1	6
13	1	1	0.5	0	0.5	0,5	1	1	1	6,5
14	1	1	0.5	0	1	0,5	1	1	1	7
15	1	1	0.5	0	0.5	0,5	1	1	1	6,5
16	1	1	0.5	1	1	0,5	1	1	1	8
17	1	1	0	1	1	0,5	1	1	1	7,5
18	0	1	0	0	1	1	1	1	1	6
19	0	0.5	0	0	1	0,5	1	1	1	5
20	1	1	0	0.5	0.5	0,5	1	1	1	6,5
21	0.5	0	0	0	1	0,5	1	1	1	5
22	0	0	0	0	1	0,5	1	1	1	4,5
23	1	1	1	1	1	0,5	1	1	1	8,5
24	0	1	0	0	1	0,5	1	1	1	5,5
25	0	1	0	0	0	0,5	1	1	1	4,5
26	1	0	0	0	0	0,5	1	1	1	4,5
27	0	1	1	1	1	0,5	1	1	1	7,5
28	0	0	0	0	1	0,5	1	1	1	4,5
29	1	1	1	0.5	1	0,5	1	1	1	8
30	0	0	0	0	0	1	1	1	1	4
31	0	0	0	0	0	1	1	1	1	4
32	1	0	0	0	1	0,5	1	1	1	5,5
33	1	0.5	1	0	1	0,5	1	1	1	7
34	0	1	0	0	1	1	1	1	1	6
35	0	1	0	0	1	1	1	1	1	6

36	1	1	1	0	1	1	1	1	1	8
37	0	1	0.5	0.5	1	1	1	1	1	7
38	1	1	0.5	0	0.5	1	1	1	1	7
39	1	1	0.5	0	0.5	1	1	1	1	7
40	1	1	0.5	0	0.5	1	1	1	1	7
41	1	1	0	0	0	0,5	1	1	1	5,5
42	1	1	0	1	1	1	1	1	1	8
43	1	1	0	0	0	1	1	1	1	6
44	0.5	1	1	0.5	1	1	1	1	1	8
45	1	0	0	0	0	1	1	1	1	5
46	1	1	1	0.5	1	0,5	1	1	1	8
47	0.5	1	0	0	0	1	1	1	1	5,5
48	0.5	1	0	0	0	1	1	1	1	5,5
49	0.5	1	0	0	0	1	1	1	1	5,5
50	0	1	0	1	1	1	1	1	1	7
51	0	1	0	0	1	1	1	1	1	6

Table A3: Bacterial isolate identification.

ISOLATE NUMBER	ACCESSION NR	% IDENTITY SIMILARITY	IDENTIFICATION	SUBSTRATE
14	NR_074841	96.92%	Aeromonas hydrophila strain ATCC 7966	HDPS
16	NR_074841	96.92%	Aeromonas hydrophila strain ATCC 7966	LDPE
17	NR_029252.1	96.18%	Aeromonas caviae strain ATCC 15468	LDPE
23	NR_041019.1	96.39%	Stenotrophomonas koreensis strain TR6-01	Nylon
27	NR_104824.1	96.05%	Aeromonas caviae strain CECT 4221	HDPS
29	NR_112116.2	96.96%	Bacillus subtilis strain IAM 12118	W
33	NR_041019.1	96.39%	Stenotrophomonas koreensis strain TR6-01	Nylon
36	NR_074841	96.92%	Aeromonas hydrophila strain ATCC 7966	HDPS
37	NR_113602.1	96.09%	Brevundimonas diminuta strain NBRC 12697	HDPS
38	NR_074841	96.92%	Aeromonas hydrophila strain ATCC 7966	HDPS
39	NR_074841	96.92%	Aeromonas hydrophila strain ATCC 7966	HDPS
40	NR_074841	96.92%	Aeromonas hydrophila strain ATCC 7966	HDPS
42	NR_042415.1	94.64%	Providencia vermicola strain OP1	HDPE
44	NR_112838.1	95.86%	Aeromonas veronii strain JCM 7375	HDPE
46	NR_042413.1	96.59%	Providencia rettgeri strain DSM 4542	W
50	NR_113602.1	96.09%	Brevundimonas diminuta strain NBRC 12697	W