

Characterization of microbial assemblages on micro-plastics in a river system

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ABSTRACT

Microplastics as pollutants originate from land-based sources and are persistent and ubiquitous in the aquatic environment. The risks posed by microplastics as vectors and habitats for potentially harmful microbial groups and antibiotic resistance genes (ARG) are relatively unexplored in South Africa. The relationship between microplastics and microorganisms' reels in the threat of creating superbugs that may proliferate resistance profiles of potentially pathogenic bacteria in the environment. This study aimed to characterize the bacterial community composition (BCC) and quantify the ARG on microplastics from freshwater settings. The first objective was to analyze the process of microbial colonization on microplastics and wood substrates through Scanning Electron Microscopy (SEM) using microcosm experiments of river water and wastewater treatment effluent (WWTe). This was followed by next generation sequencing (NGS) targeting the 16S rRNA gene to characterize the BCC on the substrates and surrounding water (river and WWTe) post-incubation. The third objective was to isolate microplastics and collect water samples from across five sites from the Mooi River, followed by the identification of the microbial consortia by 16S rRNA metabarcoding of the environmental DNA samples. The fourth objective was to investigate the prevalence of the *int1* gene and six AmpC β -lactamase gene groups, including ARG copy numbers (AmpC gene groups) on all microplastics, wood, and water samples. The microcosms' studies showed that substrates can be colonized within 48 hours after incubation. Moreover, the surface properties of the substrates and the nutrient load in water play a role in the process of colonization. SEM images revealed biofilm development in river water is slower than in nutrient-rich water (WWTe) and from environmental microplastic samples, the images revealed a diverse microbial community of bacterial biofilms and eukaryotic organisms inhabiting the surfaces. Two hundred and six (206) microplastics were isolated from 1800 litres of surface running water following *in situ* filtrations through 300-75 μ m pore size metal sieves at each site. Bacterial diversity indices of both microcosm and environmental samples showed that the BCC on microplastics was less rich, diverse, and even than in the surrounding water. Furthermore, the bacterial profiling showed that microbial phyla dominance fluctuated during the incubation period. The BCC dominance on microplastics from the environment differed from that of the surrounding water at each respective site. LEfSe biomarker analysis showed that the families *Aeromonadaceae*, *Enterobacteriaceae*, and *Legionella* were significantly higher ($p < 0.05$) on microplastics than in water samples, including some of the genera known to contain potentially pathogenic members which show resistance against antibiotics. Conventional PCR revealed that the *int1* gene was present in WWTe samples from the microcosm experiments and in environmental samples that contained more than five AmpC β -lactamase gene groups. Overall, real-time qPCR revealed that ARG/16S rRNA gene copy

numbers were greater in microplastics than in water samples from the river. However, the gene copy numbers in WWTe water samples were greater than in microplastic samples. The study revealed that the abundant colonization, selective enrichment of potential pathogenic microbial groups, and high ARG/16S rRNA gene copy numbers on microplastics displayed microplastics as possible vectors and hotspots for gene transfer between pathogenic and non-pathogenic microbial groups. Moreover, coupled with the durability of microplastics, wastewater treatment plants (WWTP) can introduce wastewater-related pathogens into microplastic biofilms which can be dispersed to downstream parts of the river.

Keywords: ARG, Bacterial community composition, Microplastics, Microcosm, NGS, River, 16S rRNA

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LIST OF ABBREVIATIONS

ARB	Antibiotic Resistant Bacteria
ARG	Antibiotic Resistant Gene/s
β	Beta
BCC	Bacterial Community Composition
COD	Chemical Oxygen Demand
DEA	Digital Elevation Dataset
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
DTI	Department of Trade and Industry
DWAF	Department of Water Affairs and Forestry
ESβL	Expended Spectrum Beta Lactamase
ESKAPE	<i>Enterococcus faecium</i> , <i>Staphylococcus aureus</i> , <i>Klebsiella pneumoniae</i> , <i>Acinetobacter baumannii</i> , <i>Pseudomonas aeruginosa</i> , and <i>Enterobacter spp</i>
ESRI	Environmental Systems Research Institute
FISH	Fluorescence in situ hybridization
FTIR	Fourier-transform infrared spectroscopy
HGT	Horizontal Gene Transfer
HDPE	High-Density Polyethylene
LEfSe	Linear discriminant analysis Effect Size
LDA	Linear Discriminant Analysis
LD-PE	Low-density Polyethylene
NASA	National Aeronautics and Space Administration
NFEPA	National Freshwater Ecosystem Priority Areas
NGS	Next Generation Sequencing
NWADC	North West Department of Agriculture, Conservations and Environment
NWP	North West Province
NWU	North-West University
PAH	Polycyclic Aromatic Hydrocarbons
PCR	Polymerase Chain Reaction
PE	Polyethylene
PP	Polypropylene
PS	Polystyrene

PVA	Polyvinyl alcohol
qPCR	Quantitative Polymerase Chain Reaction
rRNA	Ribosomal Ribonucleic Acid
SA	South Africa
SANBI	South African National Biodiversity Institute
SEM	Scanning Electron Microscope
SoER	State of the Environment Report
USA	United States of America
WHO	World Health Organization
WMA	Water Management Areas
WWTe	Wastewater Treatment effluent
WWTP	Wastewater Treatment Plant/s

CHAPTER 1: INTRODUCTION

1.1 General introduction

The mass production of plastic that occurred in the early 1930s and 1940s introduced one of the most essential materials that became part of our daily lives (Jambeck *et al.*, 2015). Plastics are widely used across industries because of their long-term durability and low-cost efficiency. Despite the beneficial use of plastics, these polymers have become one of the most challenging pollutants in aquatic systems due to the harmful impact on marine biota (Oberbeckmann *et al.*, 2014). Microplastics (< 5 mm) originating from large plastic items and those primarily produced in plastic manufacturing facilities (Verster *et al.*, 2017; Li *et al.*, 2018) are highly abundant in freshwater systems. It is reported that microplastics are globally distributed in the aquatic environment (Li Y *et al.*, 2020), and most of the marine systems are contaminated by microplastics that may have harmful toxicants and residues (Zettler *et al.*, 2013; Oberbeckmann *et al.*, 2014; Arias-Andres *et al.*, 2018a). Furthermore, these polymer fragments may come directly from freshwater systems associated with WWTe and end up in marine settings (Jambeck *et al.*, 2015; Yokota *et al.*, 2017; Harrison *et al.*, 2018; Luo *et al.*, 2019).

1.2 The relationship between microorganisms and microplastics

According to Rummel *et al.* (2017) and Arias-Andres *et al.* (2018a), microplastics in the aquatic system can form associations with other pollutants in the water column such as organic matter and chemical substances including heavy metals, antibiotics, and other xenobiotics through adsorption (Karami *et al.*, 2016). The growing concern from a microbiological point of view is that microplastics can be colonized by various microbial cells that maintain stability through biofilm formation on the substrates (Zettler *et al.*, 2013). Studies have reported that the microbial consortia on microplastics can gain and exchange genetic material and host pathogenic and non-pathogenic microorganisms (Keswani *et al.*, 2016; Arias-Andres *et al.*, 2018a; Eckert *et al.*, 2018). Microplastics can be transported over long distances in the water column and can pass through wastewater treatment systems. It is estimated that about 83-95% of microplastics are removed in WWTP (Dris *et al.*, 2015). This suggests that the smaller fragments that are not sufficiently removed from the influent can be released back into freshwater systems (rivers, dams, and lakes) together with wastewater-related microorganisms (Estahbanati and Fahrenfeld, 2016; Murphy *et al.*, 2016; Harrison *et al.*, 2018). Kirstein *et al.* (2016) found *Vibrio parahaemolyticus* (a potentially pathogenic bacterium) on floating microplastic particles found in the North and Baltic Sea. The interactions that occur between microplastics and microorganisms suggest that microplastics act as

vectors for pathogenic microorganisms and aid in the transfer of resistance genes between different species on the microplastic biofilms (Arias-Andres *et al.*, 2018a). These may be ingested by a range of aquatic organisms from phytoplankton to zooplankton, fish, crustaceans and eventually humans (Keswani *et al.*, 2016; Ogonowski *et al.*, 2018).

1.2.1 The link between microplastic biofilms and antibiotic resistance

The battle to combat antibiotic resistance has gained momentum in the research field especially since the detection of high levels of antibiotic resistance genes (ARG) in freshwater systems (Kümmerer, 2009). There is evidence that within microplastic biofilms, there is an exchange of ARG via mobile genetic elements between microorganisms. For example, Arias-Andres *et al.* (2018a), demonstrated that there was an increase in the frequency of plasmid transfer between bacterial cells on microplastics through their microcosms' studies. Members of the *Enterobacteriaceae* family were found to be in abundance in microplastics found in water samples (Oberbeckmann *et al.*, 2018). The family *Enterobacteriaceae* consists of several genera such as *Escherichia*, *Klebsiella*, *Enterobacter*, and *Citrobacter*. These genera contain clinically relevant species and wastewater-related organisms also known to harbour various resistance and virulence genes (Bergeron *et al.*, 2015). Antibiotic usage and resistance development are closely interlinked and antibiotic resistance in the *Enterobacteriaceae* family in clinical settings has been reported in South Africa (Bezuidenhout *et al.*, 2017).

1.3 Problem statement

South Africa is a water-scarce country (Coetzee *et al.*, 2016) in which the increase in human population growth including economic and industrial development has contributed to the deterioration of the natural freshwater system in terms of water quality (Jordaan and Bezuidenhout, 2016). Freshwater systems such as dams, rivers, and lakes are exposed to anthropogenic activities directly and indirectly. Domestic sewage and WWTe are typically the main direct pollution sources for urban rivers because they contain high concentrations of nutrients, xenobiotics, and heavy metals, including macro- and microplastics (Li *et al.*, 2012). Studies have reported on the presence of microbial activity on microplastics (Hossain *et al.*, 2019; Mu *et al.*, 2019). However, more information and studies on the structure and function of microorganisms colonizing microplastics in freshwater systems are needed, especially in South Africa.

1.4 Aim and objectives of the study

The aim of the research on microplastics is to account for the interactions between diverse plastic materials undergoing weathering and colonization by microorganisms in a freshwater

system. The study sought to provide a science-based risk assessment for the effects of plastic debris in aquatic environments. Therefore, the investigation and analysis of the “plastisphere” (Zettler *et al.*, 2013) will assist in gaining functional insight into its productivity and diversity as well as its vector role in carrying and dispersing wastewater-related bacteria and ARG. This study aimed to detect and characterize microbial assemblages on microplastics in a South African river system, with the following objectives:

- To conduct microcosm studies to investigate biofilm formation on substrates visually using SEM.
- To characterize microbial consortia on microplastics in WWTe versus river water.
- To characterize the microbial consortia on microplastics isolated from the river system.
- To detect and quantify ARG from microplastic biofilms and water samples.

CHAPTER 2: LITERATURE REVIEW

2.1 Water in South Africa

South Africa is characterized as a semi-arid to arid country with a rainfall pattern that is irregular and seasonal, averaging 450 mm/year (Botai *et al.*, 2018). The country depends on three resources for water: groundwater, surface water, and return flow. Surface water is a major source of water that is utilized for urban, industrial, and irrigation (Sinha and Kumar, 2019). According to Stats SA (2018), 2.5% of water is directed to mining, 3% for industrial use, 2% goes to power generation, 6% is utilized by agriculture and 27% is left for consumption for a population of over 60 million. Although access to potable water in some households has improved, 2.7% of the population still collects water directly from rivers, dams, and wells (Masindi and Duncker, 2016). Factors such as population growth, pollution and drought in South Africa have mounted a lot of pressure on the demand of freshwater resources that are suitable for consumption (Oberholster and Ashton, 2008).

2.2 Water challenges in the North West Province

The North West Province (NWP) is faced with many challenges regarding water availability and quality. The surface water system consists of rivers, dams, and wetlands which are impacted by anthropogenic activities such as mining, domestic, and industrial sewage effluent, agriculture, and livestock farming (Jordaan *et al.*, 2019). Additionally, people use the surface running water for religious practices and recreational activities (Zenani and Mistri, 2005; NWADC-SoER, 2008). For management purposes, the water resources in South Africa are regionalized into 19 Water Management Areas (WMA), including approximately 824 WWTP facilities across the country (Mitchell *et al.*, 2014). The NWP has four WMA categorized into the Upper Vaal, Middle Vaal, Lower Vaal, and the Crocodile (West) Marico. Water and sanitation facilities are generally prone to mismanagement and mechanical fault in some parts of the province. Thus, it is important to formulate strategies that will sustain these facilities to ensure safe and clean water is provided (Masindi and Duncker, 2016). The changes in nutrient load brought upon by anthropogenic disturbances into the water column affect the biogeochemical cycles driven by bacterial communities, leading to the deterioration of the overall water quality (Bricheux *et al.*, 2013; Jordaan and Bezuidenhout, 2016). Several studies in the NWP have demonstrated that clinically relevant bacteria and wastewater-related organisms are persistent in the river system and are released together with ARG from the WWTP (Njage and Buys, 2015; Jordaan and Bezuidenhout, 2016; Molale-Tom and Bezuidenhout, 2020).

2.2.1 Consequences of microplastic pollution in relation to WWTe in South Africa

The function of WWTP is to treat polluted water and return it to the environment in an acceptable quality (Mitchell *et al.*, 2014). The discharge of poorly treated or untreated wastewater in the environment poses a health risk to humans, especially those who are immunocompromised (Raju *et al.*, 2018). WWTP facilities do not facilitate the removal of microplastics. Consequently, microplastics as emerging pollutants provide a perfect habitat for microbial agents, xenobiotics, and chemical agents in the water column that escape filtering processes in WWTP (Murphy *et al.*, 2016). The escape may be accelerated and proliferated by sewage influent and storm-water runoff during overload, most especially in non-performing wastewater treatment facilities, as this has been observed in densely populated areas (Oberholster and Ashton, 2008). There is limited research in South Africa on the interactions between microplastics and microorganisms in freshwater and WWTP systems (Keswani *et al.* 2016; Bouwman *et al.*, 2018; Oberbeckmann *et al.* 2018; Eckert *et al.*, 2018).

We need to understand the complex interactions between microbial assemblages and microplastics in water as well as their ability to sustain biofilms on various macro- and microplastics if the resilience of aquatic systems to microplastic pollution is assessed (Rummel *et al.* 2017). Considering pollutant control Luo *et al.* (2019) expressed that it is important to trace the source and behaviour of microplastic from terrestrial ecosystems to freshwater systems. The type of river (tributary and/or the main river channel) and the surrounding area where the river flows, has an impact on the quality of water. The pollution sources, anthropogenic impact, and hydrodynamics can potentially influence the rates and accumulation of microplastics in aquatic systems. The flow dynamics of WWTe carrying plastic debris allows for a further increase in the levels of microplastics in freshwater systems and further dispersal into other parts of the river (Browne *et al.*, 2011).

2.3 Plastic consumption in South Africa

Plastics are widely used across the global economy. The plastic manufacturing industry contributes about 1.9% to the gross domestic product and contributes to the economic growth employment rate in South Africa through manufacturing and recycling companies across the supply chain (Verster *et al.*, 2017). According to the Department of Trade and Industry (DTI), in 2017 the plastic consumption in South Africa was approximately 1 450 000 metric tons of virgin plastics with about 334 727 metric tons of recycled input material, which indicates a per capita consumption of 28 kg. About 55% is used for packaging, whereas 15% is for building

and construction material. Other sectors such as engineering, agriculture, automotive, and domestic products use about 6% or less of plastic material (Verster *et al.*, 2017).

For years, plastic bags in South Africa were issued for free by supermarkets and clothing stores for transporting and packaging of goods. During this period, South Africa consumed about 8 billion plastic bags, which were initially non-recyclable. Due to improper waste management and irresponsible discarding of plastic bags, which caused blockages of gutters and drain systems, a levy charge on plastic bags was introduced in 2003 by supermarkets (Dikgang *et al.*, 2012). The levy charge was meant to encourage consumers to reuse plastic and reduce plastic pollution. Since the emergence of plastics as a pollutant, numerous actions have been taken to ensure that plastic pollution is reduced. Plastic SA introduced and launched awareness campaigns urging the reuse and recycling of plastic material (Plastic SA, 2018). However, this seemed to address the pollution of larger plastics items. The impact of microplastics is still an underlying issue that has proven to be problematic in the aquatic system and needs further investigation.

2.4 The cycle of microplastics as contaminants in the aquatic system

2.4.1 What are microplastics?

Plastic debris in the environment is a cause for concern, especially in the aquatic environment. The complex malleability and non-biodegradable properties of the plastic material make it environmentally persistent as an aquatic contaminant (Horton *et al.*, 2017; Jiang *et al.*, 2018). Microplastics are described as polymers with a size class of less than 5 mm (Bouwman *et al.*, 2018), and they can be classified into two groups: primary and secondary microplastics. Primary microplastics are substrates that are manufactured to be microscopic, usually termed microspheres or microbeads (Wu *et al.*, 2019). They are vastly produced for industrial and household products such as hand-cleaners and facial scrubs (Figure 1: A). Secondary microplastics (Figure 1: B) are substrates that originate from large plastic materials due to fragmentation. Fragmentation occurs when large plastic items are exposed to environmental factors such as photo-degradation and turbulence from the wave actions in the water body causing the plastic item to break down into smaller pieces (Keswani *et al.* 2016). Microplastics are present in different shapes and textures such as fragments, fibres, and spheres (Yan *et al.*, 2019).

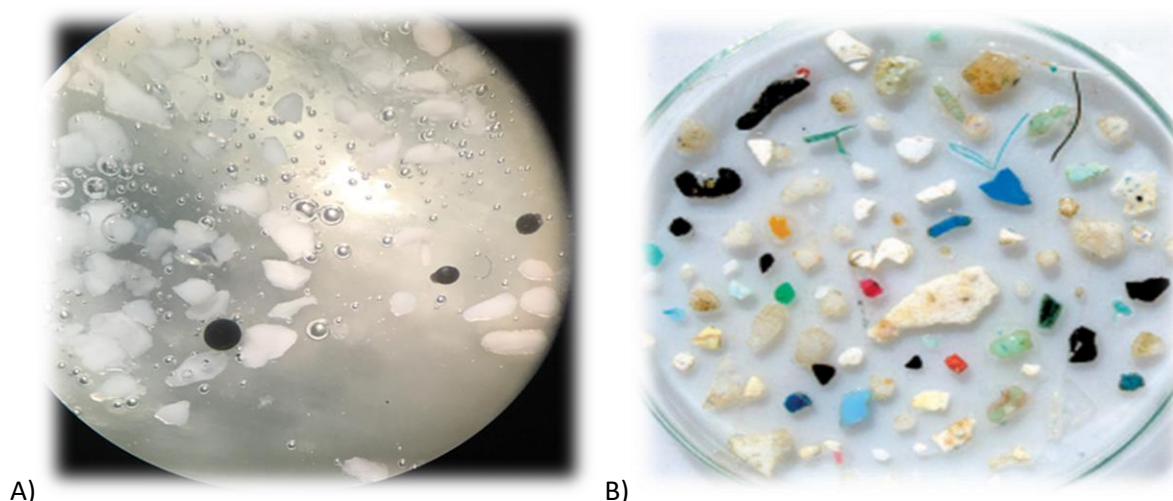


Figure 1: Images showing the two groups of microplastics. A) Primary microplastics from a facial scrub visualized under a stereomicroscope. B) An example of secondary microplastics which may originate from large plastic items such as plastic bottles, synthetic clothes, and plastic bags (Abreu and Pedrotti, 2019)

2.4.2 The sources and fate of microplastics in the aquatic environment

The presence and accumulation of microplastics in the marine environment as pollutants is well investigated (Zettler *et al.*, 2013; Nel and Froneman, 2015; Bryant *et al.*, 2016). It is reported that most macroplastics and microplastic debris originates from the terrestrial environment which is washed into freshwater systems (Jambeck *et al.*, 2015; Lebreton *et al.*, 2017; Li Y *et al.*, 2020; Verster and Bouwman, 2020). The sources include: (a) Illegal dumping sites followed by improper disposal of waste and unmonitored waste disposal sites (Figure 2), (b) household and cosmetic products containing microbeads, (b) industrial facilities producing plastic material, and (c) effluent discharges from WWTP which directly pollute riverine systems with plastic debris (Bouwman *et al.*, 2018; Lin *et al.*, 2018; Verster and Bouwman, 2020). As per Figure 2 (1) Macroplastics undergo (2) fragmentation to form microplastics, and within that entry, there is (3) biofilm formation which may contain viruses, pathogenic, and non-pathogenic microorganisms (Rummel *et al.*, 2017). Microplastics are less dense than water, thus, they float in the water column and their density decreases as biofilms form (Li, C *et al.*, 2020). Eventually, these microplastics are either (4) ingested by organisms or settle down at the bottom. Once ingested by organisms they can release plasticizers and adsorbed biological toxins and chemicals in the food chain leading to biological health effects (Rummel *et al.*, 2017; Jiang *et al.*, 2018)

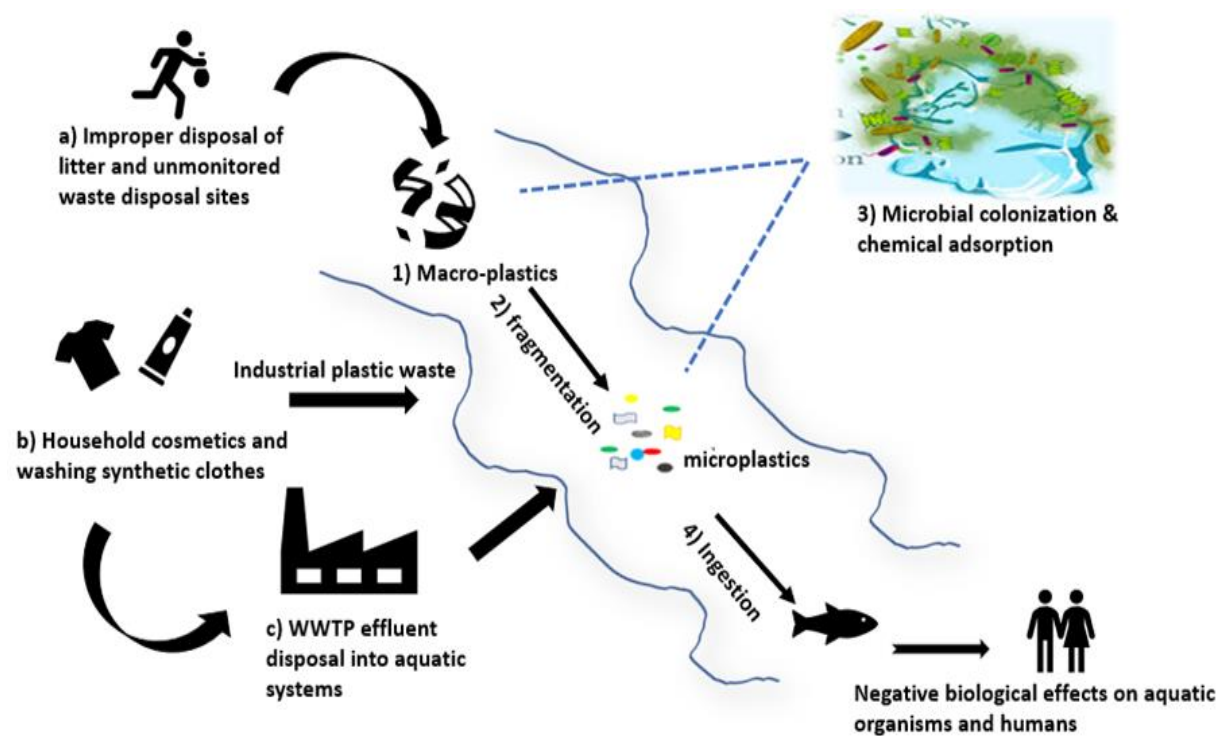


Figure 2: Illustration of the possible source and fate of microplastics in the riverine system (Rummel *et al.*, 2017; Rochman and Hoellein, 2020).

2.4.3 Microplastics and microbial biofilms interactions

Generally, microorganisms are present in the aquatic environment. They may exist in a unicellular form or as biofilms attached to a natural solid surface such as rocks and wood (Douterelo *et al.*, 2018), with an arrangement of colonies and cellular and extracellular polymeric substances (Flemming *et al.*, 2016; Miao *et al.*, 2019). Biofilms are a composition of diverse communities of bacteria, algae, protozoans, and fungi that are phylogenetically and functionally different from each other (Rummel *et al.*, 2017). It has been demonstrated that biofilms are of great importance to microbial functions and ecological processes in water (Battin *et al.*, 2016). Cardinale *et al.* (2002) stated that biofilm formation is influenced by the surrounding environmental conditions, including the type and properties of the surface they associated with during the early stages of biofilm formation (Oberbeckmann *et al.*, 2018). Microplastics were initially thought to be inert until biofouling organisms (hydroids and diatoms) were identified harbouring on industrial pellets found in the Sargasso Sea (Carpenter and Smith, 1972 in Nel and Froneman, 2015). The study of Carpenter and Smith conducted in 1972 is believed to be the first report on the detection of hydroids and diatoms attaching to plastic surfaces, suggesting that plastic materials can support or provide a suitable surface for biofilm development (Laganà *et al.*, 2019).

2.4.4 The role of WWTP on the dispersal of biofilms attached to microplastics in freshwater

Freshwater systems can be contaminated directly by microplastics in one of three ways: a) through WWTe discharge, b) overflow of wastewater sewers during the high rain season and c) run-off from sludge applied to agricultural land (Eriksen *et al.*, 2013). WWTP are not designed to fully remove microplastics (Kay *et al.*, 2018). WWTP remove between 83-95% of microplastics (Dris *et al.*, 2015). Other studies have reported that some WWTP can remove between 97-98% of microplastics (Talvitie *et al.*, 2015). However, there is still a substantial quantity of microplastics being released with the effluent. The study of Mintenig *et al.* (2017) revealed that approximately 9×10^3 pieces of microplastics per m^{-3} were being released with the effluent in a German WWTP. They further reported that, of the twelve WWTP they investigated, a total discharge of up to 4×10^9 microplastic particles and fibres per WWTP were released into the receiving water body. Based on reports that microplastics support the formation of biofilms (Raju *et al.*, 2018), this could serve as a route of escape for wastewater-related microorganisms when microplastics are released into freshwater (Parrish and Fahrenfeld, 2019).

WWTP could play a significant role in introducing wastewater and clinically related pathogenic microorganisms such as the ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter spp*) pathogens during biofilm build-up on microplastics (McCormick *et al.*, 2014; Mulani *et al.*, 2019). WWTP do not fully remove antibiotic residues. Therefore, they provide a medium for the development of resistance mechanisms for microorganisms against antibiotics (Baquero *et al.*, 2008). This results in the exchange of resistant genes between pathogenic and non-pathogenic resistance genes carrying bacteria in water and within microplastic biofilms (Pham *et al.*, 2021). WWTP influence the fate of ARG and ARB. It has been reported that a significant amount of cell-free ARG in the effluent showed the potential of turning into a pollutant after disinfection (Manzetti and Ghisi, 2014; Zhang *et al.*, 2018). For example, a study found the presence of ARG conferring resistance to β -lactams and methicillin in phage DNA, isolated from a WWTP and water samples downstream the WWTP (Colomer-Lluch *et al.*, 2011).

2.5 Health impact of microplastics on aquatic biota

2.5.1 Microplastics in the aquatic food web

Given the prevalence of microplastics in aquatic systems, the likelihood of microplastics entering food webs is expected. At different trophic levels, microplastics are prone to ingestion

by suspension feeders and filter feeders like protozoans, rotifers, mussels, and crustaceans (Browne *et al.*, 2008). For example, a South African study by Naidoo *et al.* (2016) found microplastic fragments in the stomach contents of the estuarine mullet (*Mugil cephalus*) in KwaZulu-Natal Durban harbour. It is reported that the concentration of microplastics and the non-selective feeding mode of the fish in the estuarine may have contributed to the amount of plastic ingestion by the fish (Naidoo *et al.*, 2015). Colonization of microplastics by microbes and the adsorption of organic and inorganic matter may enhance the nutritional value of the substrates, making them attractive for some aquatic biota. Bacterivorous and herbivorous ciliates, including bivalves, have been reported to effectively feed on microplastic beads carrying bacterial cells (Harrison *et al.*, 2018). Indirect ingestion of microplastics via prey to predator is also likely to occur in aquatic food webs, resulting in the transfer of microplastics from lower to higher trophic levels in the food chain (Scherer *et al.*, 2017; Li B *et al.*, 2020).

2.5.2 Effects of microplastics on aquatic biota

Freshwater biota such as mussels and benthic invertebrates accumulate microplastics mainly from sediments, while microplastics in non-benthic fish stomach contents are primarily from floating microplastics in water (Li B *et al.*, 2020). Microplastics have been shown to accumulate in the gills and digestive tracts of marine crustaceans and fish, which may interfere with the feeding process of the organisms (Jin *et al.*, 2018; Wang *et al.*, 2019; Yuan *et al.*, 2019). However, there are reports that some organisms such as the goldfish (*Carassius auratus*) can excrete microplastics rapidly. Therefore, there is no accumulation within their guts (Grigorakis *et al.*, 2017), demonstrating that some organisms can remove microplastics with quick succession. Adsorbed chemicals from the surrounding environment and chemicals from plastics such as phthalates and bisphenol A, classified as endocrine disrupters, have the ability to affect the life cycle parameters of some species (Oehlmann *et al.*, 2009). Laboratory experiments have shown a reduction in algal growth after 78 days exposure to polypropylene (PP) and high-density polyethylene (HDPE) fragments (Largade *et al.*, 2016). However, Canniff and Hoangs' (2018) study observed an increase in algal growth in the presence of polyethylene (PE) beads, which served as substrates for the algae.

2.6 Physico-chemical impact on microplastic biofilms

2.6.1 Environmental factors' role in biofilm development on microplastics

Upon entry of plastic debris into the aquatic environment, the first action is the formation of a coating layer of inorganic and organic substance on the surface of the substrates through

adsorption (Rummel *et al.*, 2017). The coating layer allows for microorganisms to come into contact with the plastic surface through repulsive and attractive forces facilitated by cell walls and enzymatic actions which may involve other biomolecules and proteins (Figure 3) (Renner and Weibel, 2011). It is worth noting that in environmental settings, microbial colonization may have occurred before the substrates entered the aquatic body. Once the plastic item is discarded, it is exposed to harsh environmental conditions such as ultraviolet radiation, abrasions, and fragmentation from hydrodynamic wave actions, which occur as the polymers travel across the aquatic system. This phenomenon is described as weathering (Rummel *et al.*, 2017). This may serve as a chemo-signal for specific microbial attachment to the polymers in water (Restrepo-Flórez *et al.*, 2014). Additionally, successive weathering and microbial colonization on the plastic material will change the particles' surface topography, density, vertical position in the water column (Barnes *et al.*, 2009).

2.6.2 Biotic factors influencing biofilm formation on microplastics

The surface and type of plastic materials plays a role in supporting bacterial colonization (Hossain *et al.*, 2019). Although plastic materials are inert (Mason *et al.*, 2016), synthetic polymers are known to contain different compositions of additives and chemicals that are added to improve the materials' properties (Koelmans *et al.*, 2016). Some of the factors enhancing microbial colonization on the plastic material include surface roughness, hydrophobicity (Rummel *et al.*, 2017), and biofouling. Biofouling may lead to the deterioration of macroplastics to microplastics creating a rougher surface on the microplastics and may open the door for colonization by other organisms on the substrates (Zardus *et al.*, 2008; Hossain *et al.*, 2019). The interactions that occur between microorganisms and the substrate allows for numerous adaptation strategies such as surface charge and hydrophobicity of the cell walls (Hori and Matsumoto, 2010; Krasowska and Sigler, 2014). This promotes a change in the cell's membrane. The presence of cell surface structures such as pili, flagella fimbriae may improve adhesion across the microplastic surface (Renner and Weibel, 2011; Rummel *et al.*, 2017). Once the initial coating and the first colonizers and/or microbial cells have settled, the initial material surface is modified enabling other organisms to attach, be it on the available plastic surface or the layer of the first colonizers (Lobelle and Cunliffe, 2011).

A study conducted by Oberbeckmann *et al.* (2018) showed that some environmental factors played a role in facilitating microbial cell attachment on microplastic and wood substrates. Furthermore, they reported that surface parameters were secondary role players in the early stages of colonization. Additionally, factors such as pH, nutrients ionic strength, and COD (Chemical oxygen demand) are reported to induce enrichment of microbial cells for biofilm formation on microplastics (Renner and Weibel, 2011). The first step in biofilm formation is the

adhesion and adsorption of microbial cells on the substrate (Figure 3). Hydrophobic interactions involving the cells' surface structures influence attachment to the surface (Renner and Weibel, 2011). Eventually, the surface becomes modified, allowing for the expression of extracellular polymeric substances. The attached first colonizers act as quorum sensors and/or chemo-signals for other microorganisms to induce biofilm formation (Subramani and Jayaprakashvel, 2019). The surface topography and chemical contents of the substrates, such as plastic polymers influence the behaviour of microbial colonization to the substrates.

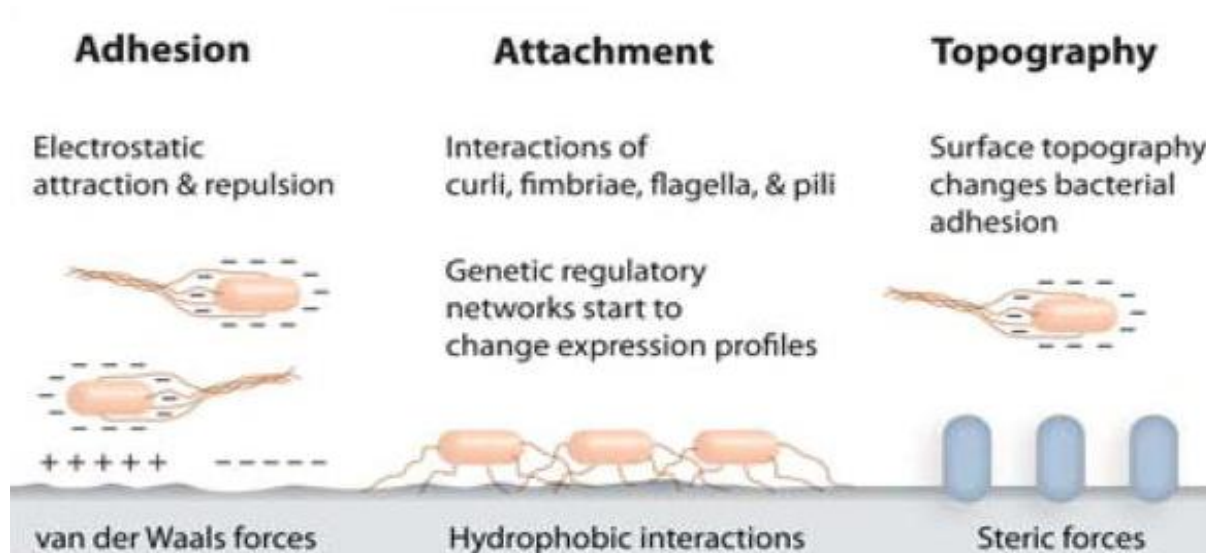


Figure 3: Illustration of the process and factors influencing microbial attachment to substrates (Renner and Weibel, 2011).

2.7 Microbial activity and diversity on microplastics

2.7.1 Diverse microbial activity on microplastics in water

Microorganisms within the “plastisphere” (an ecosystem of microbial biofilms on the microplastic surface) (Zettler *et al.*, 2013) may perform complex metabolic functions and biogeochemical activities (Madigan *et al.*, 2014). For example, it was found that the heterotrophic activity increased due to biofilm formation on microplastics, which resulted in the alteration of the carbon cycles (Arias-Andres *et al.*, 2018b). Nitrogen and Phosphorus cycling processes can also be altered by the microplastic-microorganism interactions in water (Chen *et al.*, 2020). The presence of bacterial sequences belonging to the family *Erythrobacteraceae* on microplastics has been reported. Members of this family are known to produce carotenoids and bacteriochlorophyll, which are beneficial for biogeochemical cycles (Tonon *et al.*, 2014; Oberbeckmann *et al.*, 2018). The primary colonizers, through various chemotaxis mechanisms, can shape the community that is to form a matured biofilm. A study by Bryant *et al.* (2016) found that secretion system genes; *che* and *nifH* may enhance chemotaxis, nitrogen

fixation, and cell-to-cell interactions in microplastic biofilms (Oberbeckmann *et al.*, 2014; Oberbeckmann *et al.*, 2018). The genus *Erythrobacter* is reported to have enzymatic properties that can degrade polycyclic aromatic hydrocarbons (PAH), which may be present on plastic polymers (Gao *et al.*, 2015). Reports have also detected a high abundance of sequences belonging to the genus *Pseudomonas* on microplastics compared to non-plastic substrates. Some members of *Pseudomonas* are known to degrade polymers such as PP, polyethylene (PE), and Polyvinyl alcohol (PVA) (Shimao, 2001; Kathiresan, 2003; Oberbeckmann *et al.*, 2018)

2.7.2 Prevalence and dispersal of potential pathogens on microplastics

Research studies suggest that microplastics select for distinct bacterial assemblages, which differ in taxonomic composition from other natural substrates such as water, glass, wood, and rock (McCormick *et al.*, 2014; Besemer, 2015; McCormick *et al.*, 2016; Wu *et al.*, 2019). The selective enrichment may depend on the specific characteristics of the substrate such as the type of polymer, surface properties, and chemical composition (Kießling *et al.*, 2015). The detection of potentially pathogenic genus *Vibrio* on polypropylene particles in the marine environment revealed that microplastics provide habitat for pathogenic microorganisms (Zettler *et al.*, 2013). Due to the hydrodynamic flow of river water, dispersed microplastic biofilms containing pathogenic species may change the microbial community's structure and function in the surrounding water (Wu *et al.*, 2019). For example, a study by Masó *et al.* (2003) reported that the outbreak of the harmful algal bloom *Alexandrium taylori* was potentially introduced and further dispersed by plastic debris which had *A. taylori* cells attached to the debris. WWTP may also shape the microbial community by introducing wastewater-associated bacteria as the plastic debris passes through the treatment system. This was evident in the study of Oberbeckmann *et al.* (2018), where they found sequences belonging to the *Enterobacteriaceae* family specifically on PE and PS particles from samples downstream from the WWTP.

2.8 Antibiotic resistance and microplastics

2.8.1 Sources of antibiotics in the aquatic environment

Antibiotic resistance has become a global health concern due to the prevalence and occurrence of antibiotic-resistant microorganisms in clinical and environmental settings (Marti *et al.*, 2014). Antibiotics are prescribed and used in clinical, agricultural, and veterinary settings to treat and prevent a wide variety of bacterial infections (Kümmerer, 2009) (Figure 4). Due to the partial metabolization of antibiotics, residues in the excreta are secreted as waste and enter sewage systems together with the human, animal, and soil microbiota into WWTP and

river streams (Baquero *et al.*, 2008; Vaz-Moreira *et al.*, 2014). Freshwater systems affected by anthropogenic activities are considered hot spots which enable and drive the development and selection of antibiotic-resistant microorganisms (Knapp *et al.*, 2011; Su *et al.*, 2015; Hu *et al.*, 2016; Imran *et al.*, 2019). Due to the dependence on surface water for irrigation, drinking, household activities, recreational and religious activities, the redistribution, and reuse of surface water leaves a great potential for ARG and ARB to become predominant in the environment (Zenani and Mistri, 2005).

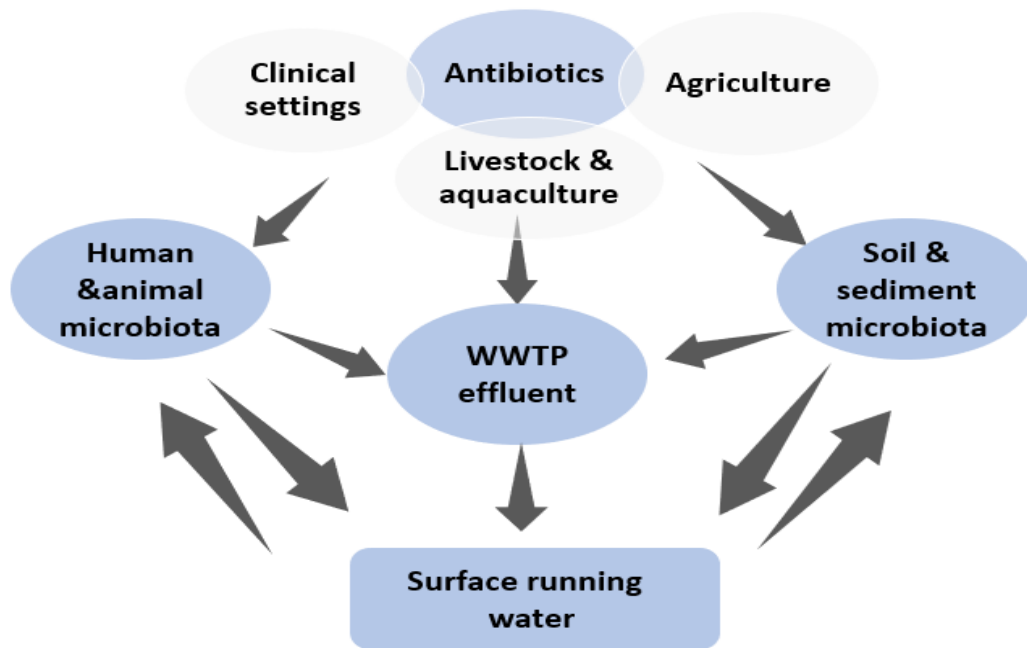


Figure 4: Illustrations of the route and linkage between systems that aid in the proliferation and transfer of ARG and ARB. The recontamination and redistribution via drinking water and irrigation increase the stability of ARG and leaves a great potential for ARB to rebound and become predominant strains in the environment (Baquero *et al.*, 2008).

2.8.2 The fate of ARG and ARB in the water environment

Freshwater habitats are amongst the natural habitats that harbour the richest bacterial diversity. Surface water serves as a significant pathway for disseminating bacteria and exchanging genetic material between different taxa in different environmental compartments (Baquero *et al.*, 2008; Zhang *et al.*, 2009). For example, bacteria and mobile genetic elements carrying genes can be mobilized between unclean water (wastewater, ponds, and rivers) to pristine water environments (disinfected and spring drinking water) and eventually to animals and humans (Marti *et al.*, 2014; Vaz-Moreira *et al.*, 2014). Although most of the antibiotic metabolites are removed during the wastewater treatment cycle, the little amount that escapes through the WWTe exposes bacteria to low toxic levels enabling them to develop resistant

mechanisms (Manzetti and Ghisi, 2014). Studies have reported the presence of ARG and ARB in drinking water (Moore *et al.*, 2010; Bai *et al.*, 2015; Bergeron *et al.*, 2015; Huijbers *et al.*, 2015). Additionally, extended-spectrum β -lactamases (ES β L) and AmpC β -lactamase genes in association with pathogenic *E.coli* have been detected in irrigated water and lettuce (Njage and Buys, 2015). This indicates that water treatment facilities are not sufficiently removing these agents from the water.

A report revealed that a few bacterial organisms belonging to the same bacterial lineages inhabiting aquatic systems could be detected in the human-associated microbiome (<http://www.hmpdacc.org/catalog/> in Vaz-Moreira *et al.*, 2014). Another metagenomic platform study found that ARG in non-pathogenic soil bacteria had a similar identity to ARG from many diverse human pathogens (Forsberg *et al.*, 2012). The hypothesis is that when humans and animals consume contaminated drinking water or food, a change in bacterial resistance profiles of their microbiota is likely to occur (Zeng *et al.*, 2019). However, studies on this theory are required to explain further the transmission and/or migration of ARG to humans. A study by Poirel *et al.* (2005) found that environmental bacteria and the same bacterial organism from clinical settings had similar resistance gene profiles. For example, the *qnrA* gene, known to confer resistance to quinolones, was identified in chromosomes of Gram-negative algae species *Shewanella algae*, which is abundant in freshwater and marine environments.

2.8.3 AmpC β -lactamase genes

β -lactams are one of the oldest and commonly used antibiotics for the treatment of infectious diseases in humans but are also used in veterinary and agricultural practice for the prevention of infections (Henriques *et al.*, 2006; Jacoby, 2009; Zhang *et al.*, 2009; Singtohin *et al.*, 2010). The emergence of antimicrobial resistance is regarded as a global health concern, especially since research studies detected the presence of ARG in microbial communities from natural environments (Chee-Sanford *et al.*, 2001; Ash *et al.*, 2002; Henriques *et al.*, 2006; WHO, 2017). However, the studies on antibiotic resistance mechanisms in the environment are at a minimum particularly for AmpC β -lactamase genes and related bacterial carriers (Coertze and Bezuidenhout, 2019). Bacteria have developed mechanisms and/or strategies to generate immunity against the effects of antibiotics (Farrokhazar *et al.*, 2016). Some of the mechanisms utilized by bacteria to confer resistance to β -lactam antibiotics include inaccessibility and alterations of the target enzymes or the production of β -lactamase enzymes, which hydrolyze and inactivate the β -lactam ring (Li *et al.*, 2007; Zhang *et al.*, 2009; Alaybeyoglu *et al.*, 2015; De Angelis *et al.*, 2020).

AmpC β -lactamases and associated plasmid-mediated genes can confer resistance to several broad-spectrum β -lactam antibiotics, including 1st, 2nd, 3rd, and 4th generation cephalosporins,

penicillins, and cephamycins, and are usually not inhibited by clavulanic acid or other inhibitors (Jacoby, 2009; Rojs *et al.*, 2019). The genes that encode for AmpC's are derived from chromosomal *ampC* genes of various members from the *Enterobacteriaceae* family (Ye *et al.*, 2017). According to Jacoby (2009), AmpC β -lactamases are inducible, and through mutation, at prominent levels, they can potentially be overexpressed. Overexpression in clinical isolates such as *Enterobacter cloacae*, *Escherichia coli*, *Citrobacter freundii*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Serratia marcescens* confers resistance to therapeutic drugs such as cefoxitin, cefotetan, and monobactams (Farrokhniazar *et al.*, 2016). The overexpression occurs either through deregulation of the *ampC* chromosomal gene or by the acquisition of transferable genetic elements such as plasmids, transposons, and integrase-integron gene cassettes harbouring the *ampC* gene (Pérez-Pérez and Hanson, 2002; Geyer *et al.*, 2012). The AmpC β -lactamases that are plasmid-mediated include the CMY family, FOX family, MOX-1; -2, ACT-1, ACC-1, MIR-1, DHA-1; -2, BIL-1 and LAT-1 to LAT-4 group types (Pérez-Pérez and Hanson, 2002; Jacoby, 2009; Farrokhniazar *et al.*, 2016).

2.8.4 Procurement of antibiotic resistance genes

Antibiotic resistance may occur through multiple complex mechanisms which have evolved through mutations and selection. For example, when microbes encounter antibiotic stress they may prohibit the entry of the antibiotic through the cell membrane. Additionally, they can reduce the sensitivity of the cellular target and modify their intracellular structure and/or deactivate the antibiotic (Taylor *et al.*, 2011). However, other microbial cells may not survive in the process. Microorganisms undergo various genotypic and phenotypic changes in response to environmental pressure (Brooks, B., and Brooks, A., 2014). Their ability to adapt to changes in the environment, especially in unfavourable conditions such as antibiotic stress, results in the development of mutations and selection within their genetic compositions (Jordaan and Bezuidenhout, 2016). Naturally, antibiotic-producing microbes have resistance genes that protect them against lethal doses of synthesized antibiotics. Other microorganisms develop resistance through exposure to antibiotics or by acquiring these resistance genes from other microorganisms through horizontal gene transfer (HGT) (Forsberg *et al.*, 2012; Imran *et al.*, 2019). HGT of resistance genes can occur through various processes (Figure 5) in which foreign DNA derived from a donor cell is introduced into a recipient cell predominantly via (a) phage mediated transport (transduction); (b) by uptaking mobile genetic elements from the extracellular environment (transformation) or (c) by direct intercellular exchange (conjugation) (Drudge *et al.*, 2012).

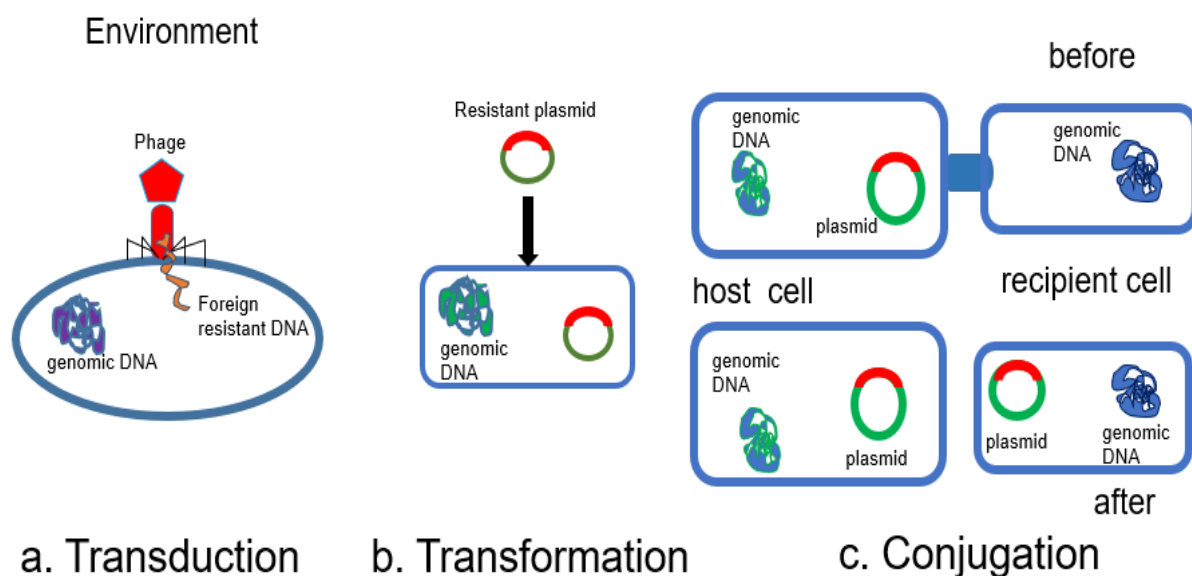


Figure 5: Mechanisms of horizontal gene transfer (Drudge *et al.*, 2012; Imran *et al.*, 2019).

2.8.5 Fundamental elements aiding in genetic exchange

Mobile genetic elements such as plasmids play an important role in transmitting ARG between different microorganisms in the environment. Transposons and integron-integrase elements do not self-replicate and are either carried by phages or plasmids, enabling them to move from cell to cell (Zhang *et al.*, 2009). Insertion sequences and transposons can occasionally jump on bacterial genome or plasmids and may result in resistance to various agents (Naas, 2007). Integron-integrase genes have recombinant features which enable the incorporation of multiple resistance genes, expression in their variable regions, and transmission by conjugative plasmids (Figure 6) (Gillings *et al.*, 2014; Vaz-Moreira *et al.*, 2014). Under a stress-enhanced environment resulting from pollutants (antibiotic residues, faecal matter, heavy metals, wastewater, and agricultural runoff), the potential for co-selection of resistant genes is highly induced (Di *et al.*, 2016). The drive for its co-selection is activated due to the physical location of integrons and transposons on plasmids, which carry ARG.

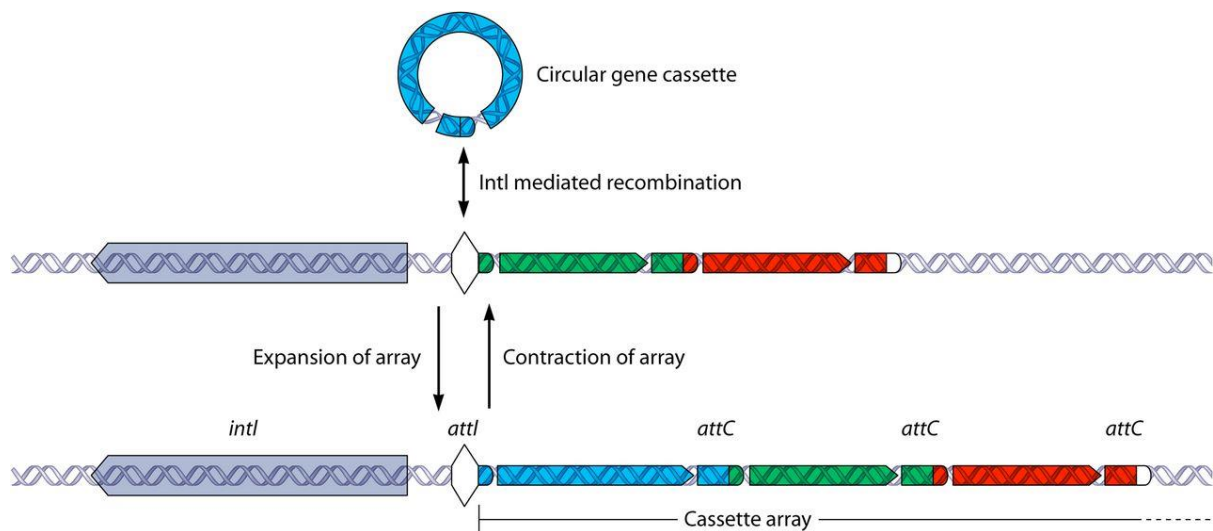


Figure 6: Schematic diagram of the integron-integrase structure and how it incorporates gene cassettes into its' genome. The *intl* site can catalyze the recombination between the site of circular gene cassettes (*attC*) and the attendant integron recombination site (*attI*) (Gillings, 2014).

Integrins-integrases are modular structures that can incorporate and express various exogenous resistance genes (Figure 6). Their structure allows an arrangement of ARG that can be transferred simultaneously so that the selection of one ARG co-selects other resistance genes within the integron (Martinez, 2009). Their characteristics also enable them to be incorporated and transferred by different plasmids into commensal and non-pathogenic bacteria (Vaz-Moreira *et al.*, 2014). ARG may be clustered in the same gene cassettes with other elements, which provide microorganisms with an ecological advantage (Japoni *et al.*, 2008). Studies and reviews have been written on the presence and evolution of integrase genes in clinical research and on their transition from clinical settings to the environment, including the correlation between class 1 integrase genes (*intl1*), which have been used as an anthropogenic pollution marker due to its presence in polluted environments (Chen *et al.*, 2013; Gillings *et al.*, 2014). Furthermore, the *intl1* gene has also been detected in areas where multiple ARG and metal resistance genes have been found (Di *et al.*, 2016; Wright *et al.*, 2008; Coertze and Bezuidenhout, 2018). This suggests that the integrins-integrase genes may facilitate the communication and dissemination of resistance genes via HGT (Jiang *et al.*, 2018).

2.8.6 Microplastics' role in driving antibiotic resistance

Microplastics are reported to be new substrates for biofilms. With a rarely explored feature, they can potentially become hotspots for exchanging ARG by HGT (Arias-Andres *et al.*, 2018a). Conjugation is considered as the main mechanism of directed HGT in the plastisphere

due to close contact of microbial cells and nutrient availability which allows for rapid exchange of genetic material in the biofilms (Aminov, 2011; Drudge and Warren, 2012). This process can occur even between distantly related taxa, affecting bacterial evolution and the spread of multiple phenotypic traits, such as ARG or heavy metal resistance genes (Carattoli, 2013). Ideally, in WWTP, the rate and frequency of mutation in bacteria within biofilms may be generally higher compared to planktonically growing isogenic bacteria which may also result in increased gene transfer. The physiological conditions and external environmental conditions may shape the biofilm composition as they inhabit microplastics substrates. Furthermore, bacterial cells in biofilms may simultaneously produce enzymes that degrade antibiotics and overexpress efflux pumps, inhibiting the effects of the antibiotics (Høiby *et al.*, 2010; Singh *et al.*, 2017). This may explain why biofilm bacteria may become multidrug-resistant against antibiotics such as β -lactams, aminoglycosides, and fluoroquinolones (Molin and Tolker-Nielsen, 2003; Driffield *et al.*, 2008). Taking into consideration that biofilms attached to microplastics substrates are transported to various parts of the river, this is worrisome.

2.8.7 Interactions between antibiotic residues and microplastics

Macroplastics and microplastics have been proven to adsorb various organic pollutants such as aromatic hydrocarbons (Rochman *et al.*, 2013), biphenyls (Velzeboer *et al.*, 2014), and antibiotics (Li *et al.*, 2018). This provides an advantage for microorganisms harbouring on microplastics to develop resistance mechanisms against antibiotics. Li *et al.* (2018) reported that adsorption capacities of antibiotics to microplastics varied amongst different types of antibiotics (sulfadiazine, amoxicillin, trimethoprim, and tetracycline) and plastic types under different environmental conditions (i.e., ionic strength and pH). They further observed a higher rate of recurrence of adsorption of antibiotics to microplastics in freshwater than in seawater. This may be due to the concentration of antibiotic residues in freshwater considering the occurrence of faecal and sewage contamination and antibiotic usage and pollution from households near the river streams. Another study by Yu *et al.* (2020) observed that the adsorption amount of tetracycline antibiotics on PE particles increased as the particle size decreased compared to other particles. Furthermore, it was demonstrated that aged microplastics have a higher sorption affinity for antibiotic residues. Antibiotics independently degrade slowly in the environment. In combination with pollutants, their bioaccumulation and combined toxicity may become a health threat to aquatic animals and humans through the food cycle and microbial community (Guo and Wang, 2019).

2.8.7.1 The link between ARG and bacteria on microplastics

The migration pattern of ARG and ARB from the aquatic system to humans is quite complex. Exposure of plastic-attached microorganisms to pollutants such as chemicals and pharmaceuticals affects the antibiotic resistance profiles of the biofilm community on microplastics (Laganà *et al.*, 2019). Bacteria and phage mediated ARG dispersion were shown to be stimulated by sophorolipid in soil contaminated by microplastics, harbouring tetracycline residues (Sun *et al.*, 2018). The study of Yang *et al.* (2019) showed that the ARG profile from macroplastics and microplastics DNA samples differed from the surrounding water where microplastics were isolated. Their investigation revealed that 17/17 ARG were present in all plastics samples, but only 4/17 ARG were detected in seawater samples. Shannon-Wiener indices indicate that the richness of ARG and multi-drug resistance genes found in microbiota on microplastics was greater than those resistance genes found in seawater microbiota. This demonstrates that plastics are serving as a reservoir of ARG in aquatic systems. Studies revealed that the distribution and profiles of ARG on these polymer substrates were largely shaped by the bacterial communities (Yang *et al.*, 2019; Sun *et al.*, 2021).

A study conducted by Eckert *et al.* (2018) reported that with increased concentration of microplastics in their microcosms, there was also an increase in the relative abundance of the *int1* gene and 16S rRNA gene copies isolated from microplastics. Due to the proximity of microbial cells on microplastic biofilms, it can be deduced that this may trigger the frequent mobilization of mobile genetic elements to other microorganisms, even those without the resistance genes (Gillings *et al.*, 2014). The accumulation and exposure of non-resistant and non-pathogenic microorganisms on microplastics biofilm and clinically relevant pathogenic microorganisms may create an opportunity for the sharing of genes, which results in environmental superbugs (Adegoke *et al.*, 2017; Wang *et al.*, 2021). More studies are still required to understand the impact of microplastics carrying ARG and ARB in freshwater, particularly in drinking water, to analyze the potential risks on humans.

2.9 Methods for detection and characterization of microorganisms on microplastics

Detecting and identifying microplastic particles in the aquatic environment is quite complex, and researchers apply different techniques to achieve their study objectives (Klein *et al.*, 2018). While there is no standard method for sampling and quantifying microplastics in aquatic systems, several studies have been published on various methodologies used to identify microplastics in the marine settings (Kirstein *et al.*, 2016), freshwater systems (Li *et al.*, 2018), sediments (Sarijan *et al.*, 2018), and soil (Sun *et al.*, 2018). The discovery of microbial life on

plastic debris using SEM provided the first glimpse of what is known as the “plastisphere” (Carpenter and Smith, 1972 in Zettler *et al.*, 2013). However, isolation methods for detecting microplastics and identifying the function, structure, and composition of biofilms on microplastics with the use of SEM are not adequate. Thus, researchers have various microscopy techniques coupled with *in situ* hybridization for visualization. Molecular methods ranging from genetic fingerprinting techniques to high-throughput sequencing have been applied to study the microbial community composition on microplastics (De Tender *et al.*, 2017).

2.9.1 Sampling and detection methods for microplastics and biofilms in freshwater systems

A wide variety of studies have been conducted with different sampling methods, treatment of samples, quantification, and identification of microplastics in the aquatic environment. However, these methods have not been standardized (Bouwman *et al.*, 2018). According to Hidalgo-Ruz *et al.* (2012) for aquatic settings, sampling methods that can be applied can either be volume-reduced (reducing the volume of the sample during the sampling period) or bulk sampled (no water volume is reduced). Microplastics are less dense than water (Zettler *et al.*, 2013). A trawl along a transect could be used to collect water samples at the surface of the water body, with the placement of a flowmeter to measure the flow rate of water to determine the total volume of water collected (Free *et al.*, 2014; Rocha-Santos and Duarte, 2015). The type of mesh size of nets used is dependent on the study’s detection limits of capture and the type of aquatic system being sampled. For example, the National Oceanic and Atmospheric Administration (NOAA) of the United States of America (USA) suggested mesh size is 333 μm (Masura *et al.*, 2015). Some researchers have used the 80 μm mesh sieve, which has resulted in microplastic samples 100 000 times higher than the 333 μm mesh sieves (Lozano and Mouat, 2009). Another approach of collecting microplastics in freshwater (rivers or dams) is to use hand-net sampling, which usually requires a steel hand bucket to collect a certain volume of surface water followed by hand-net filtration (Moore *et al.*, 2011; Bouwman *et al.*, 2018).

The challenge with methods for investigating biofilms on microplastic samples is that the tools used to isolate and identify microplastics cannot be applied to characterize biofilm composition on the polymers (Parrish and Fahrenfeld, 2019). Methods that have been used to isolate and identify microplastics from water samples involve visual picking and filtration of microplastics (Bouwman *et al.*, 2018). These are followed by incubation in density separation solutions, oxidation to remove organic matter and other contaminants, and drying of samples after removal of biofilms (Masura *et al.*, 2015). Fourier-transform infrared spectroscopy (FTIR) is usually applied to identify the polymer type of the samples, and this requires the removal of all

impurities on the microplastics to limit interference with the surface scans (Kirstein *et al.*, 2016; Bouwman *et al.*, 2018). It is recommended that biofilms be extracted from microplastics before FTIR analysis. However, this opens a door for scrutiny of visual inspection, which may lead to misclassification and identification of microplastics (Parrish and Fahrenfeld, 2019).

2.10 Visualization techniques typically used for the plastisphere

2.10.1 Scanning Electron Microscopy

SEM utilizes high-resolution magnification to produce three-dimensional images to provide topographical, morphological, and compositional information of bacterial biofilms (Walker *et al.*, 2001). The use of SEM to observe plastic material assists in visualizing the composition and the spatial structure of microbial life that has colonized the substrates. In preparation for SEM, the samples are subjected to fixation, staining, drying, and conductively coating steps before high vacuum imaging (Priester *et al.*, 2007). For imaging of microplastic substrates, SEM is quite useful because images showing various morphologies of microplastic colonizers can be obtained. Also, grooves and pits on the microplastic surface can be visualized through this instrument (Eich *et al.*, 2015). Although SEM offers a detailed look at the surface of the plastic for microbial life, it has limitations in identifying taxonomic groups detected on the surface. Identifying different microorganisms up to species level using SEM alone is impossible (De Tender *et al.*, 2017). Additionally, SEM is costly and labour-intensive, and it may present challenges in automation and image quality.

2.10.2 Epifluorescence microscopy and Fluorescence *in situ* hybridization (FISH)

To identify microbial communities on plastic debris to obtain greater taxonomic detail, epifluorescence microscopy coupled with the application of rRNA-based probes using FISH can be applied to obtain information about the microbial community on the surface of plastic debris (DeLong *et al.*, 1989; De Tender *et al.*, 2017). In a study conducted by Delong *et al.* (1989), intact cells were hybridized with fluorescent-labelled 16S rRNA probes. Because of the abundance of rRNA in cells, the binding of fluorescent probes to individual cells could be readily visualized by fluorescent microscopy. Furthermore, using phylogenetic appropriate probes (labelled with different fluorescent dyes) composed of primer sequences that distinguish between the primary kingdoms (archaea, bacteria, eukaryotes) and closely related organisms could allow for the identification of different cell types in the same microscopic field. The FISH technique was also applied to investigate microbial life residing on low-density polyethylene (LD-PE) in marine seawater and sediment. The technique revealed various structures of filamentous and isolated bacteria, including diatoms and heterogeneous

distribution of eukaryotic cells from seawater samples (De Tender *et al.*, 2017). However, microplastics from sediments were dominated by prokaryotic cells (Harrison *et al.*, 2014).

The advantages of using FISH are that:

- a) it provides taxonomic information about the microbial community present on the microplastic surface,
- b) it provides visualization of the spatial structure of the community on the microplastic debris, and
- c) it can provide data about actual abundance versus the relative abundance of the target cells (Valm *et al.*, 2012).

Since the function of biological systems is often associated with the microorganisms' structure, investigating the spatial distribution of microorganisms could explain the physiology and interactions of microbial communities on microplastic surfaces (Schlundt *et al.*, 2020). The disadvantage of using FISH methods is that phylogenetic probes target and hybridize with rRNA in the cell (Zwirgmaier, 2005). This becomes a problem for communities that are not actively growing, so the fluorescent signal would be weak, and certain taxa could be overlooked. Another limitation is that FISH techniques need fluorescently labelled probes, requiring molecular knowledge of communities to be interrogated in the environment which becomes more costly and labour intensive (De Tender *et al.*, 2017).

2.11 Phenotypic and molecular techniques for characterization of microbial communities on microplastics

To apply molecular techniques for ecological studies, two questions that drive an investigation are to find out; (1) the kind and quantity of microorganisms' present and (2) the interactions between the microorganisms and the substrate and how their activities relate to the ecosystems' functions (Konopka, 2009). Laboratory cultivation of microorganisms enables the identification of microbial communities using Sanger sequencing of rRNA genes (Sabat *et al.*, 2017). The downfall with cultivation methods is that more than 99% of the microorganisms in the environment are not culturable by standard techniques for cultivating. Thus, the data is only limited to culturable microorganisms and the bulk of microbial life is left unexplored (Amann *et al.*, 1995; Stewart, 2012). Culture-independent methods make it possible for researchers to explore most of the microbial community in the environment. The molecular methods that have been previously applied to characterize and quantify microbial communities on microplastics from aquatic systems include clone libraries (Harrison *et al.*, 2014), Denaturing Gradient Gel Electrophoresis (DGGE) (Oberbackmann *et al.*, 2014), quantitative real-time polymerase chain reaction (qPCR) (Parrish and Fahrenfeld, 2019), and high-

throughput sequencing (Oberbeckmann *et al.*, 2014, McCormick *et al.*, 2014). These techniques are PCR-based and subject to PCR bias (De Tender *et al.*, 2017). However, some of these techniques have provided great insight into gathering information about most microorganisms especially those that are unculturable.

2.11.1 Clone libraries and Sanger sequencing

For environmental samples, the 16S rRNA gene is the most frequently used marker gene for taxonomic classification (Sabat *et al.*, 2017). This is due to its beneficial characteristics such as (a) it is present in all domains of life, (b) it follows the evolutionary path of a species, and (c) it contains the largest known reference database of sequence data for comparative purposes (Větrovský and Baldrian, 2013). The construction of clone libraries allows for the 16S rRNA gene to be sequenced partially or entirely using Sanger sequencing. The complete 16S rRNA gene sequence data allows for superior taxonomic or phylogenetic assignments, thus, allowing classification up to species level (De Tender *et al.*, 2017). In some cases, especially with cloning, the resulting sequence may contain parts of the cloning vector. Considering that the process of making clone libraries is rigorous and requires time, most studies sequence less than 1000 clones, leading to only a portion of the complete community richness to be screened (Konopka, 2009). This method has been applied in the study of Dang and Lovell (2009), where they aimed to assess early bacterial colonization on various surfaces found in marine water and investigate the community richness using clone libraries.

2.11.2 Quantitative Polymerase Chain Reaction

Quantitative Polymerase Chain Reaction (qPCR) allows for the amplification and simultaneous quantification of the target DNA strand, based on the principle that the initial concentration of the target gene can be estimated according to the change in amplification cycles that are required to reach a quantification threshold fluorescence signal level (Kubista *et al.*, 2006; Zhang *et al.*, 2009). The use of qPCR has been applied in microplastics studies to amplify and determine the levels of 16S rRNA genes, and ARG found in microplastics from aquatic systems (Miao *et al.*, 2019; Parrish and Fahrenfeld, 2019). The levels of expression of target genes may be measured by absolute or relative qPCR. In absolute quantification, a calibration curve using standards of known concentrations is prepared with samples or targets of unknown concentration during a single qPCR run. However, relative quantification does not require a calibration curve during the reaction. It is based on the expression levels of a target gene versus reference genes in which the reactions can be carried out separately, and the copy numbers are calculated using the reference gene quantifications (Brankatschk *et al.*, 2012). The advantage of qPCR is that it is sensitive, and it provides a wide range of quantification to the lowest copy number (Pfaffl, 2012).

2.11.3 High-throughput sequencing: amplicon sequencing and shotgun metagenomics

High-throughput sequencing led to the increased read depth for 16S rRNA marker genes. The sequencing by synthesis method is one of the frequently used high-throughput sequencing methods (De Tender *et al.*, 2017). With the amplicon sequencing approach, the genetic markers used are amplified and sequenced simultaneously for multiple environmental samples, which produce immense parallelization of the sequencing reaction (Sogin *et al.*, 2006; Liu *et al.*, 2012). The two advantages of amplicon sequencing are that through enhanced parallelization of the sequencing reactions, the cost declines extensively. Secondly, the separation of the templates is performed without transforming the fragments into a bacterial host, saving time (Metzker, 2010). However, amplicon sequencing is limited to marker regions of approximately 500 base pairs in length, which limits the ability to perform phylogenetic inference to classify unknown reads (Li *et al.*, 2015). The amplicon sequencing method was applied in the study by Zettler *et al.* (2013) to investigate microbial communities on plastic debris versus the surrounding seawater microbial community in the North Atlantic gyre. The study detected more than 1000 Operational Taxonomic Units (OTUs) on two different microplastic polymers (PE and PP).

Shotgun metagenomic sequencing is a technique that allows for the sequencing of all genes in all organisms present in each complex sample. Environmental DNA is extracted from all cells in a community. However, instead of targeting a specific genomic locus for amplification, all DNA is sheared into tiny fragments that are independently sequenced (Sharpton, 2014). This produces DNA sequences that align to various genomic locations for the numerous genomes which are found in the sample, including non-microbes (Sharpton *et al.*, 2011). The advantage of this technique is that it provides information on functional genes (De Tender *et al.*, 2017). In a study by Bryant *et al.* (2016), the researchers compared functional genes on microplastics and seawater and found a high abundance of some genes on plastics which suggested chemotaxis and cell-to-cell interactions. This can be applied to answer the two questions of ecology studies namely: who is there and what are they capable of doing?. The challenges faced with metagenomic sequence data is that they are relatively complex and large and often tricky to analyze. In addition, because of the diverse communities, most genomes are not represented by reads. As a result, two reads from the same gene may not overlap and become impossible to directly compare through sequence alignment (Schloss and Handelsman, 2008; Sharpton *et al.*, 2011).

CHAPTER 3: MATERIALS AND METHODS

3.1 Microcosm setup

In 2019 microcosms of river water and WWTe were prepared to gain insight into biofilm development and community composition on different microplastics and naturally occurring material. Water samples were aseptically collected in sterile 1 litre bottles from the Mooi River at two sampling points: River water: Site O2 (26°41'07.5"S 27°06'05.6"E) and WWTe from Site W4 (26°45'05.3"S 27°05'40.2"E) located in Potchefstroom, NWP (Figure 7). Three types of microplastics (PS, PP, and LD-PE) were selected based on their abundance and occurrence in freshwater systems (McCormick *et al.*, 2016). Wood fragments were used as a non-plastic material control. All substrates were < 5 mm in size and were sterilized in 70% ethanol and exposed then exposed to ultraviolet radiation for five minutes. Twenty-five pieces of each of the four substrates were incubated at room temperature for fourteen days in 200 ml river water and WWTe. Additionally, water without any microplastics was used as a control. These were continuously stirred with a magnetic stirrer to simulate wave and turbulent flow as in environmental settings (Parrish and Fahrenfeld, 2019).

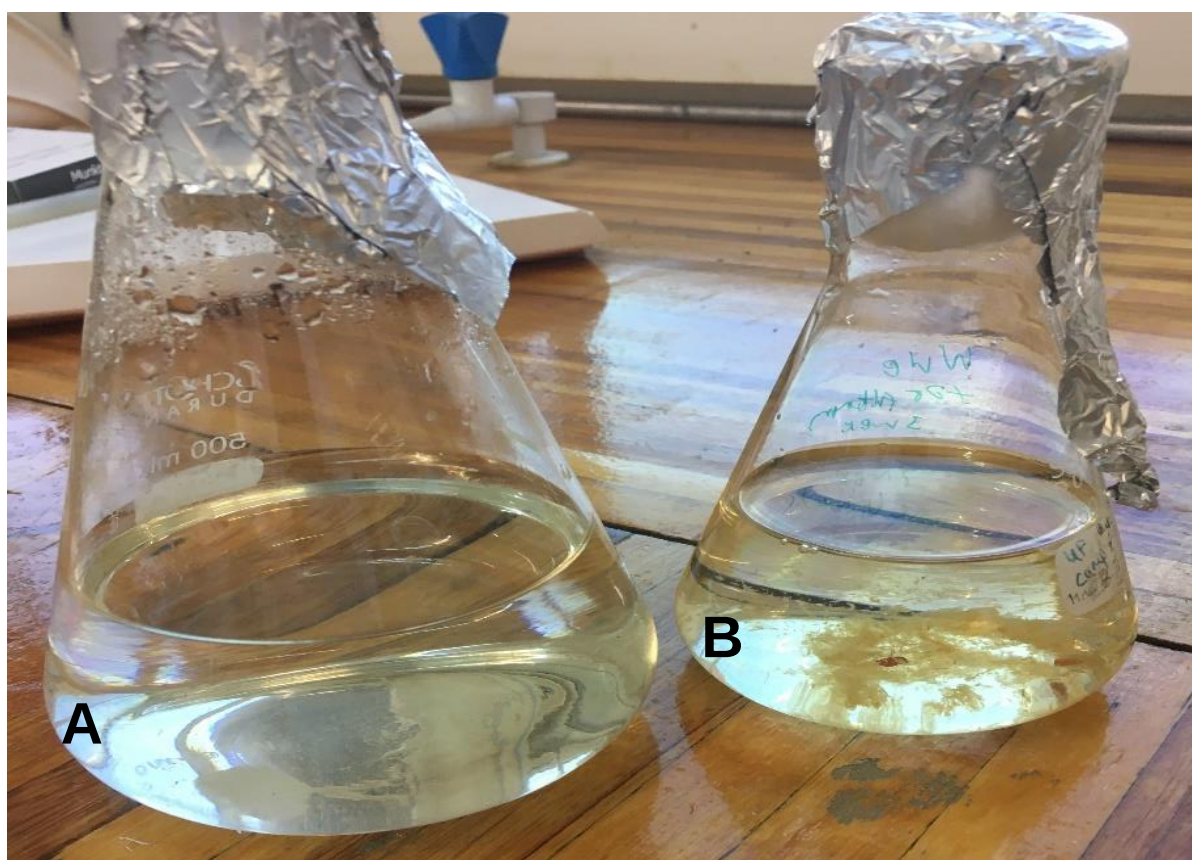


Figure 7: Examples of the microcosm vessels (post-incubation). (A) Non-substrate control vessel with only WWTe (B) Wood fragments incubated in WWTe.

One piece of each substrate was sampled aseptically from the microcosms on days two, four, seven, and fourteen to observe microbial cell colonization using SEM. After fourteen days the remaining substrates were recovered from the microcosms by filtration using a steel-sieve (25 µm). The 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 sterilized.

3.2 Environmental microplastic sampling

The method applied to isolate microplastics from surface running water was adapted from the NOAA standard microplastics protocol (Masura *et al.*, 2015; Bouwman *et al.*, 2018) with a few modifications. Sampling was conducted from September to November 2020 in the NWP Mooi River (Figure 8). All the tools and glassware used during sampling were sterilized before usage and between sampling across the different sites with 70% ethanol. A volume of 120 litres of surface water was filtered *in situ* through stacked mesh steel sieves with pore sizes of 300, 100, and 75 µm using a sterile 15 litre metal bucket which was pre- and post-cleaned to minimize contamination. A total of 1800 litres of surface water was filtered for 3 sampling runs across five sites. The collected debris on the mesh steel sieves were rinsed with distilled water and decanted into sterile glass beakers to minimize plastic contact. Surface water samples were collected in 1 litre glass bottles for analysis of the free-living microbial community. The samples were stored in ice and transported to the laboratory for further analysis.

3.2.1 Sampling sites of the Mooi River

Map creations were made with ArcGIS (10.5.1) developed by Environmental Systems Research Institute (ESRI) that is commercially available at (www.esri.com). The following data in the NWU geospatial database were used: NASA's ASTR 90m Digital Elevation Dataset; DEA: 2013 – 2014 SA National Land Cover Dataset; DWAF: Hydrology – Dams and SANBI: NFEPA River Network. Five sites were selected for sampling due to various impacts associated with land use and access to those sites (Table 1 and Figure 8).

Table 1: Map coordinates of the sampling sites where microplastics and water samples were collected.

Sample site	S	E
M1	26°29'36.3	27°07'48.5
O2	26°41'07.5	27°06'05.6
G3	26°43'32.5	27°06'19.4
W4	26°45'05.3	27°05'40.2
T5	26°45'08.8	27°06'00.9

Upstream from Potchefstroom, land-use activities include, but are not limited to, agricultural activities of crops and livestock farming. The Mooi River originates near the town of Derby and flows southwards the Gerhard Minnebron Eye (Site M1). The water quality is generally pristine, with less impact of agricultural activities and influences of the old mines from the Wonderfonteinspruit stream (Winde, 2011). Downstream the river, there is the Boskop Dam, which the population depends on for potable water. The Boskop Dam flows into the Potchefstroom Dam, initially used for irrigation but has become a popular recreation activity spot (Koekemoer *et al.*, 2021). The Mooi River flows past the Potchefstroom city (Sites O2-T5) where there is impact from agricultural and industrial activities, and recreational sites (parks, bird sanctuaries, and shopping malls). The river also flows past a WWTP (Site W4), with the effluent recycled back into the river, and the inflowing river stream joins it from the Loopspruit site after which the river enters the Vaal River.

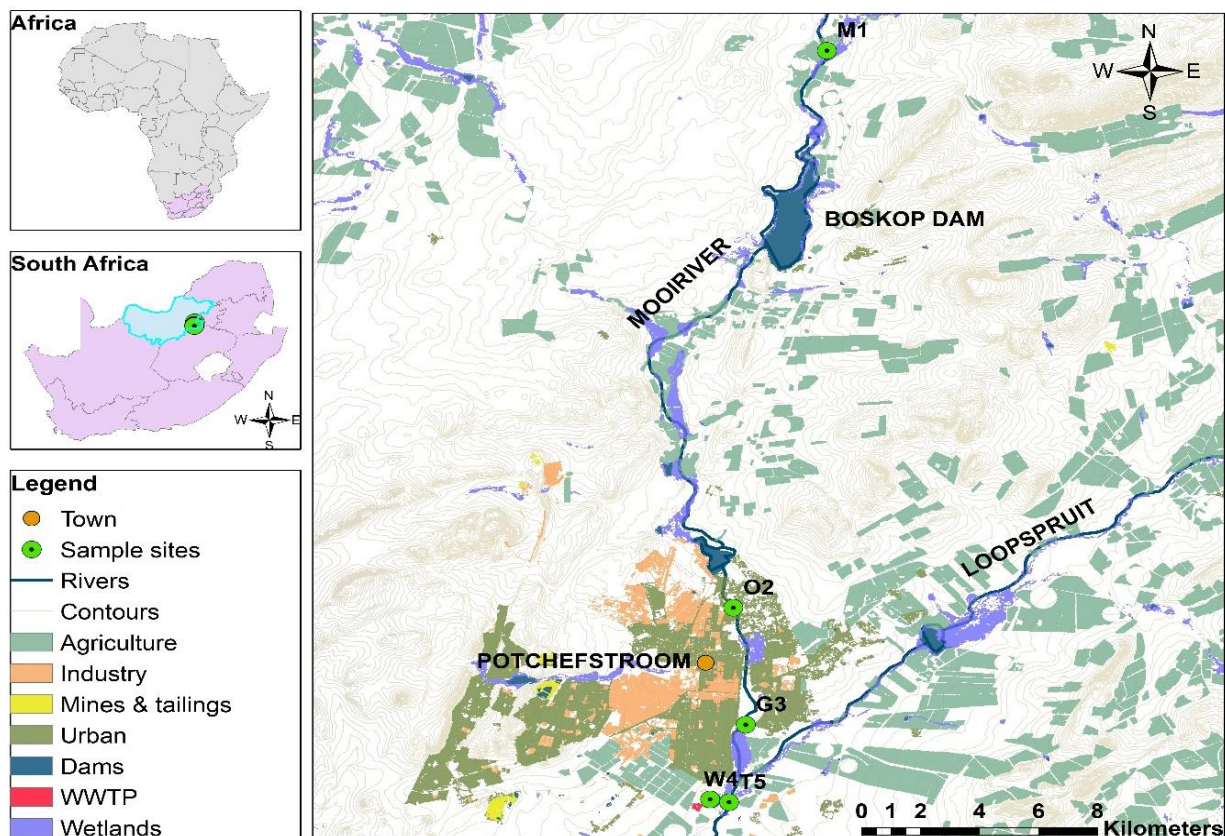


Figure 8: Map depicting the sampling sites and land cover around the Mooi River (Map created by Lohan Bredenhann).

3.3 Laboratory analysis of microplastic samples directly from the environment

Visible microplastics were aseptically collected from the debris using sterile forceps under a stereomicroscope. Based on the physical characteristics of microplastic particles (McCormick *et al.*, 2016), the samples were sorted and recorded in groups according to their classification type (fibres, films, foams, fragments, and pellets) in glass Petri dishes. The glass beakers containing field samples were filtered again through a 25 µm steel mesh and 0.45 µm nylon filter membrane in the lab. All identified microplastics were rinsed with autoclaved distilled water. Microplastics from the fragment were selected and subjected to fixation for visualization of the surface using SEM. The remaining microplastics were placed in 2 ml tubes for DNA extraction. The water samples were filtered through a 0.45 µm nylon filter membrane to extract environmental DNA. The membrane filters were cut into pieces using sterile scissors and placed in 2 ml tubes for DNA extraction. All samples prepared for DNA extraction were stored at -20 °C for further analysis.

3.4 SEM preparation

Before observations of the surface, microplastics and wood samples were fixed using 3% wt. glutaraldehyde (BDH chem. Ltd. England) in cacodylate buffer (Sigma-Aldrich), pH 6.5 for 10 minutes. The samples were exposed to an ethanol dehydration series of (50, 60, 70, 80, 96, and 2 × 100) % (v/v). This was followed by a chemical dehydration series in a 1:1 ratio of 100% ethanol and hexamethyldisilazane (HMDS, Ted Pella, USA) for 5 minutes and left to dry overnight. The samples were then sputter-coated with a palladium-gold thin film. The surfaces of the samples were viewed with a SEM/Phenom ProX desktop system (Thermo Fisher Scientific, USA) in high-vacuum mode at 15 kV (Gomes and Mergulhao, 2017) assisted by Willie Landman.

3.5 DNA extraction

DNA was extracted from microplastics, wood, and nylon filter membrane (EZ-Pak) samples using the Powersoil DNA isolation kit (Macherey-Nagel). The protocol was followed as stipulated by the manufacturer. Where applicable, the volume of reagents for microplastic samples was adjusted due to the sample size to minimize inhibition of optimal DNA extracted. The quality and quantity of the extracted DNA were determined using the Nanodrop One (Thermo Fisher Scientific, USA) spectrophotometer.

3.6 Detection of resistance genes with end-point PCR

PCR amplification of the *int11* and AmpC β -lactamase genes was conducted using the set of primers listed in Table 2. Each reaction tube contained 1 x PCR MasterMix (Thermo Fisher Scientific, USA), 0.4 μ M of forward and reverse primers, and 2 μ l (~ 1-50 ng) DNA and nuclease-free water to a final reaction volume of 25 μ l in each tube. Thermal cycling conditions for the *int11* gene were as follows; initial denaturation at 95°C for 5 minutes, 30 cycles of denaturation at 94°C for 30 seconds, annealing at 64°C for 30 seconds, and extension at 72°C for 60 seconds. The final extension step was at 72°C for 5 minutes (Labbate *et al.*, 2008). The thermal cycling conditions for amplification of AmpC β -lactamase genes were as follows; initial denaturation at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 64°C for 30 seconds for ACC, CIT, EBC, and FOX primers, the extension step was at 72°C for 60 seconds. The final extension was at 72°C for 7 minutes (Pérez-Pérez and Hanson, 2002). The annealing temperature for MOX was 59°C for 30 seconds, and the DHA annealing temperature was at 62°C for 30 seconds respectively.

Table 2: List of target genes and primers used for end-point PCR.

Target gene	Primer	Primer Sequence (5'→3')	Product size (bp)	Reference
<i>intl1</i>	HS463A	CTGGATTTCGATCACGGCACG	473	Labbate <i>et al.</i> , 2008
	HS464	ACATGCGTGTAATCATCGTCG		
ACC	ACCMF	AACAGCCTCAGCAGCCGGTTA	346	Pérez-Pérez and Hanson, 2002
	ACCMR	TTCGCCGCAATCATCCCTAGC		
MIR-1T ACT-1	EBCMF	TCGGTAAAGCCGATGTTGCGG	302	Pérez-Pérez and Hanson, 2002
	EBCMR	CTTCCACTGCGGCTGCCAGTT		
FOX-1 to Fox-5b	FOXMF	AACATGGGGTATCAGGGAGATG	190	Pérez-Pérez and Hanson, 2002
	FOXMR	CAAAGCGCGTAACCGGATTGG		
DHA-1, DHA-2	DHAMF	AACTTTCACAGGTGTGCTGGGT	405	Pérez-Pérez and Hanson, 2002
	DHAMR	CCGTACGCATACTGGCTTTGC		
LAT-1 to LAT-4, CMY-2 to CMY-7, BIL-1	CITMF	TGGCCAGAACTGACAGGCAAA	462	Pérez-Pérez and Hanson, 2002
	CITMR	TTTCTCCTGAACGTGGCTGGC		
MOX-1, MOX-2, CMY-1, CMY-8	MOXMF	GCTGCTCAAGGAGCACAGGAT	520	Pérez-Pérez and Hanson, 2002
	MOXMR	CACATTGACATAGGTGTGGTGC		

3.6.1 Agarose gel electrophoresis

The success of the PCR was confirmed by electrophoresis of the PCR products on a 1.8% (w/v) agarose gel (SeaKem, US) in 1 x TAE buffer [20 mM Acetic acid, 40 mM Tris and 1 mM EDTA at pH 8.0). The agarose gel contained 10 µl ethidium bromide (BioRad, UK). A mixture of 5 µl PCR amplification product and 2 µl 6 x orange loading dye (Thermo Fisher Scientific, USA) was loaded into each well of the gel. The fragment sizes PCR amplification products were confirmed by loading a 100 bp molecular marker (O'GeneRuler, Thermo Fisher Scientific, USA). The electrophoresis conditions were set at 100 V for 45 minutes. The gel images were captured by the ChemiDoc MP imaging system (Bio-Rad, USA) using image Lab software (version 4.0.1)

3.7 Quantification of ARG per 16S rRNA gene copies

3.7.1 Quantification of ARG: AmpC β -lactamase gene groups

Real-time qPCR was performed to determine the abundance of ARG on microplastics, specifically for clinically relevant AmpC β -lactamase gene. Following end-point PCR screening of samples for AmpC β -lactamase gene groups (ACC, ACT/MIR, BIL/LAT/CMY, DHA, FOX, and MOX/CMY), these genes were quantified using the QuantStudio™ 3 System v1.4.3 (Applied Biosystems, Thermo Fisher Scientific, USA). The qPCR reactions were performed in a total reaction volume of 10 µl. The reaction mixture consisted of 5 µl TaqMan™ Fast Advanced MasterMix (Thermo Fisher Scientific, USA), 0.5 µl of each TaqMan assay, 2 µl of eDNA, and filled to volume with nuclease-free water.

For the TaqMan gene expression assays, the following FAM fluorescent dyes were used for quantification of the various AmpC β -lactamase gene groups: Pa04646144_s1 (ACC), Pa04646124_s1 (ACT/MIR), Pa04646135_S1 (BIL/LAT/CMY), Pa04646120_s1 (DHA), Pa04646126 (FOX) and Pa04646156_s1 (MOX/CMY). Probe sequences were not made available by Thermo Fisher Scientific. Thus, product codes of the assays were provided (Coertze and Bezuidenhout, 2020). As predetermined by the QuantStudio™ 3 platform (Applied Biosystems, Thermo Fisher Scientific, USA), the thermal cycling conditions consisted of a holding stage with step 1 at 50°C for 2 minutes and step 2 at 95°C for 10 minutes. The PCR stage consisted of 40 cycles with step 1 at 95°C for 15 seconds followed by a final step 2 at 60°C for 1 minute. Standard curves were generated in ten-fold dilutions with positive control samples for each target gene containing known copies ranging from 20 000 to 2. All samples were tested independently and in duplicate, including non-template controls in the reactions.

3.7.2 16S rRNA gene quantification

Absolute quantification of the 16S rRNA gene was conducted with a known sample containing the gene of interest with a standard curve using five-fold dilution series in triplicate reactions, with copies ranging between 1200 to 1.96. The qPCR was conducted using the QuantStudio™ 3 System v1.4.3 (Applied Biosystems, Thermo Fisher Scientific, USA). Each reaction consisted of 5 µl of 2X PowerUp SYBR green MasterMix (Thermo Fisher Scientific, USA), 0.5 µl of each forward primer 906F (5'-AAA CTC AAA KGA ATT GAC GG-3') and reverse primer 1062R (5'-CTC ACR RCA CGA GCT GAC-3'), 2 µl of DNA which was filled with nuclease-free water to a final volume of 10 µl. DNA input of unknown samples was not diluted due to complexities with low concentrations from microplastic samples and the mixed bacterial nature of the environmental DNA samples. DNA input in qPCR reactions varied and was standardized for analysis as reported in the study of Coertze and Bezuidenhout (2020). The quantified copies were normalized and calculated as gene copies/16S rRNA, to understand the abundance of ARG on microplastic biofilm communities. The qPCR thermal cycling conditions were set out as follows, a hold stage of 50°C for 2 minutes followed by 95°C for 5 minutes. The PCR stage was made of 40 cycles at 95°C for 15 seconds, followed by annealing at 55°C for 15 seconds and 72°C for 15 seconds. A melt curve analysis was implemented on qPCR assays to verify primer binding to the specific target fragment. The curve was generated from 60°C to 95°C with increments of 0.5°C/cycles while collecting fluorescence data (Figure S1-A).

3.7.3 Data analysis

Microsoft Excel was used to calculate averages, R^2 values, standard deviation, and efficiency values for qPCR data, including the normalization and drawing of graphs. Amplification efficiencies of standard curves between 90% and 110% ($E = 10^{-1/\text{slope}} - 1$) and $R^2 > 0.97$ were deemed acceptable for comparison of trend lines for the quantification of genes from the samples (Coertze and Bezuidenhout, 2020). The data normalization to gene copies/16S rRNA was performed using the XLSTAT version 2105 software package (Addinsoft SARL, New York, USA).

3.8 Library preparation for 16S rRNA gene amplicon libraries and sequencing

The gene library of the 16S rRNA was prepared following the Illumina 16S metagenomic sequencing library preparation guide (Illumina, USA). Locus-specific primers 341F (5'-CCT ACG GGN GGC WGC AG-3') and 805R (5'-GAC TAC HVG GGT ATC TAA TCC-3') (Klindworth *et al.*, 2013), targeting the hypervariable V3-V4 region (approximately the 460 bp

region) of the bacterial 16S rRNA gene, were attached to Illumina forward and reverse overhang adapters (Illumina, USA) to the 5'-end of forward and reverse primers respectively. This resulted in the following PCR primers: 16S amplicon forward primer 5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG-3' and reverse primer: 5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C-3'. Briefly, the library preparation workflow entailed a first-stage amplicon PCR step aimed at amplifying the region of interest by using 12.5 ng of DNA, 12.5 µl of KAPA HiFi HotStart ready Mix [0.5 U DNA polymerase, 0.3 mM dNTPs, 2.5 mM MgCl₂] (Kapa Biosystems, Wilmington, MA, USA), 0.2 µM of each reverse and forward primers and nuclease-free water (Thermo Fisher Scientific, USA) to a final volume of 25 µl. The PCR Thermal reaction protocol was carried out as follows: denaturing at 95°C for 3 min, followed by 35 cycles of 95°C for 30 seconds, 55°C for 60 seconds, and 72°C for 30 seconds, and a final extension step at 72°C for 5 min was included.

PCR amplicons were then subjected to a PCR clean-up step using Agencourt AMPure XP magnetic beads (Beckman Coulter Genomics, USA). Following clean-up, index PCR was performed to attach dual indexes to PCR products using the Nextera XT index kit (Nextera, USA). The indexing reaction was performed using 5 µl of amplicon PCR product DNA, 5 µl of Illumina Nextera XT Index Primer 1 (N7xx), 5 µl of Nextera XT Index Primer 2 (S5xx), 25 µl of 2x KAPA HiFi HotStart Ready Mix, and 10 µl of nuclease-free water. The thermocycling conditions were 95°C for 3 minutes followed by 8 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and a final elongation step of 72°C for 5 minutes. Index PCR products were subjected to a second 'PCR clean-up' step. The partial 16S rRNA libraries were then 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. Sequencing was performed in-house using the Illumina MiSeq platform (Illumina, USA).

3.8.1 Bioinformatics and statistical analysis

Demultiplexed paired-end reads were checked for quality using FastQC software (version 0.11.5, Babraham Institute, United Kingdom). Paired reads were trimmed at both 5' and 3' ends to eliminate poor quality nucleotides, denoised, merged, depleted of chimeric sequences, and clustered into operational taxonomic units (OTU) by using the DADA2 denoiser (Callahan *et al.*, 2016) integrated into the Quantitative Insight into Microbial Ecology version 2 (QIIME2) software (Bolyen *et al.*, 2019). The denoised reads were assigned taxonomy by the Silva 16S rRNA database (Release 138) using a trained classifier of the V3-

V4 region. Thereafter, singletons and non-bacterial taxa (e.g., archaea and mitochondria) were eliminated. The output tables generated from QIIME2 were cleaned through the R programming language for the generation of the OTU table for further analysis. Downstream statistical analyses were carried out using MicrobiomeAnalyst (Dhariwal *et al.*, 2017; Chong *et al.*, 2020) online web tool with Marker Data Profiling (MDP) module (<https://www.microbiomeanalyst.ca/MicrobiomeAnalyst/home.xhtml>).

Using the MicrobiomeAnalyst for data analysis following the filtration step, abundance features and low variance features were removed based on the interquartile range. The data were normalized to the equal library size following rarefaction at over 97% good coverage. Alpha diversity indices were calculated at the genus level for richness (Chao1 estimator), diversity and evenness (Shannon index), and species dominance (Simpson index). The Linear discriminant analysis effect size (LEfSe) tool was used to identify biomarkers using the relaxed settings. Briefly, this tool applies an algorithm that determines significantly different ($p < 0.05$) taxa between groups by applying a Kruskal–Wallis test and determines condition differences via a Wilcoxon rank-sum test, then applies linear discriminant effect size analysis to reduce the dimensions (Segata *et al.*, 2011).

CHAPTER 4: RESULTS

This chapter summarises and interprets the results based on the aim and objectives outlined in Chapter 1. In this chapter, the interpretation of the results obtained from the microcosm experiments conducted in 2019 to analyze the behaviour of microorganisms during microbial colonization on microplastic and wood substrates is provided in sections 4.1 to 4.3. Section 4.4 gives an overview of the concentration of the DNA extracted from microplastics, wood, and water samples. Section 4.5 explores the microbial consortia (based on 16S rRNA metabarcoding) detected on the substrates versus filtrate water samples post-incubation. The chapter further explores environmental application in sections 4.6 and 4.7, where microplastics were isolated from the river system (with the inclusion of WWTe) to analyze and observe the bacterial community composition (BCC). Finally, sections 4.8 and 4.9 explore the prevalence of ARG, precisely six AmpC β -lactamase gene groups and the class 1 integrase (*intl1*) gene between microplastics and water, to gain insight into substrates as possible vectors of ARG in freshwater settings.

4.1 Microbial colonization on substrates

Microcosm experiments were done by incubation of sterile microplastics and wood substrates in river water and WWTe. Using SEM, the substrates were analyzed after sterilization to confirm that no other microbial cells or biofilms existed on the substrates before introducing them into the mediums (river water and WWTe). The substrates that were analyzed showed no presence of microbial cells or biofilms on all the substrates (Figure 9). The surfaces of the substrates were distinct from each other. The polymer textures selected included two solid fragments of PP and LD-PE. The polystyrene (PS) substrates were spherical and spongy, and lastly, wood fragments had different solid compartments. The remaining sterile substrates were incubated in river water and WWTe in conical flasks for microbial colonization.

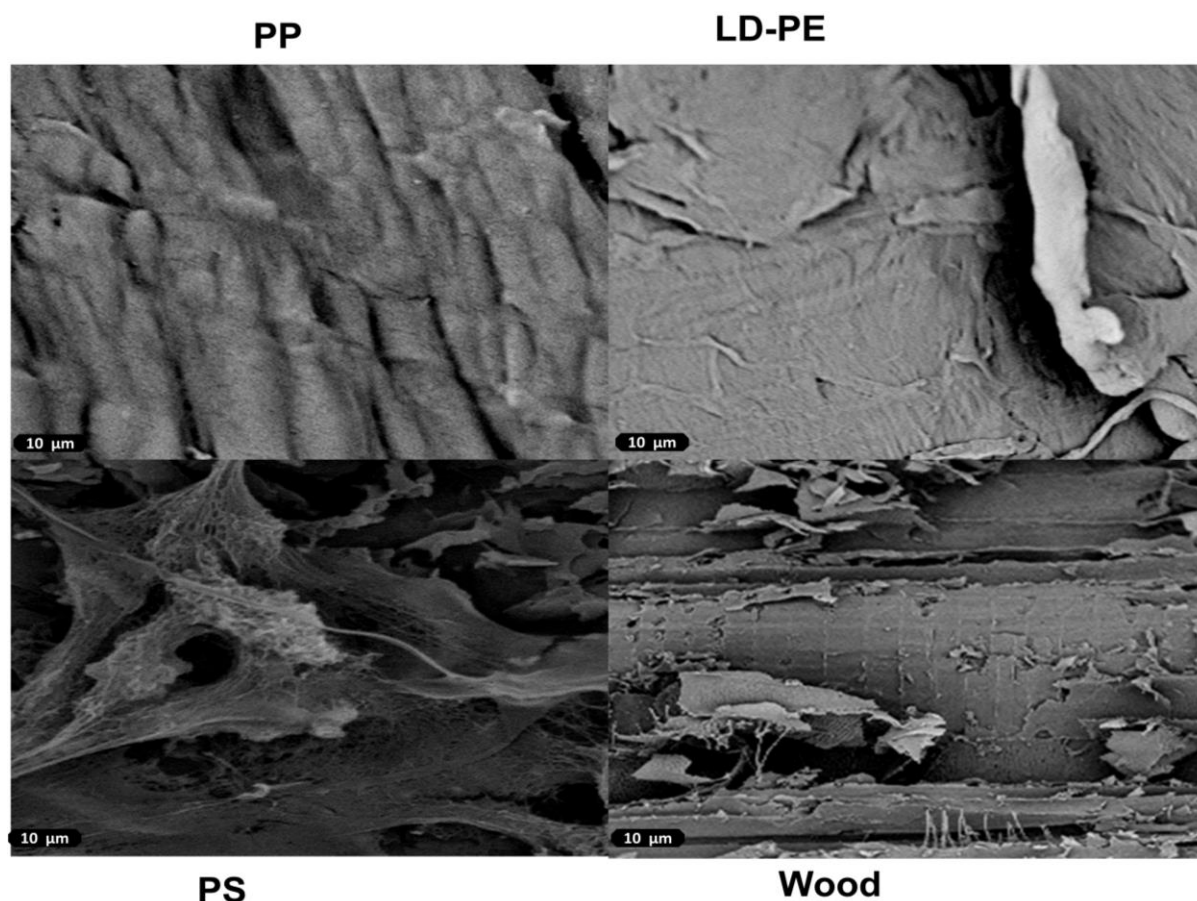
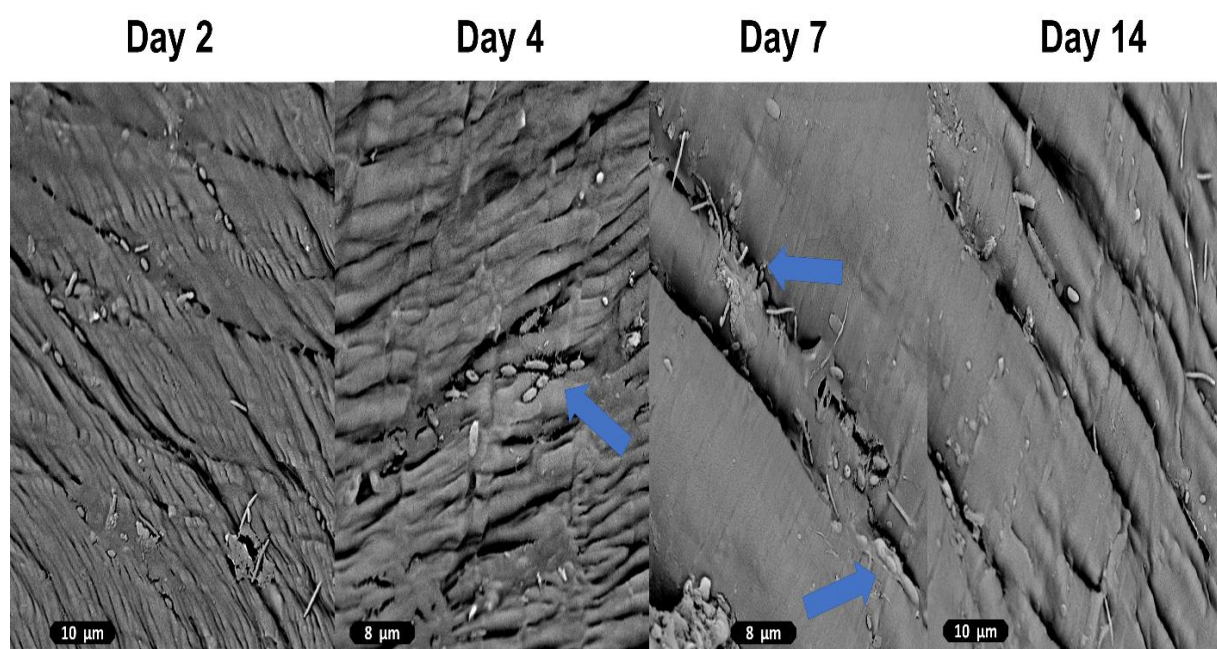


Figure 9: SEM images of microplastics and wood substrates post-sterilization viewed at a scale of 10 µm. PP (Polypropylene); LD-PE (Low-density polyethylene); PS (Polystyrene).

4.2 Microbial colonization on river microcosm substrates

The substrates were incubated in river water and isolated on different days to observe the pattern or behaviour of microbial cells when substrates are introduced. Due to the surface characteristics, it was observed that microbial cells attached more to the folded parts as opposed to the whole surface of the PP substrate on day 2 (Figure 10). Microbial cell extension structures were observed on day 4. The cells were attached to the grooves and pitting spots of the folded layers of the samples. On day 7, the clustering of cells composed of rod and round-shaped cells covered by a layer of film began to show (indicated by arrows). This suggests a formation of a premature biofilm. On the PP sample isolated on day 14, no mature biofilms were detected. However, a layer of rod cells was visible and abundant on the surface.



PP

Figure 10: Polypropylene (PP) substrates isolated from the river microcosms showing microbial colonization on the surface observed on days 2, 4, 7, and 14. SEM scale is indicated at the bottom of each image.

On LD-PE samples (Figure 11) a similar pattern of microbial colonization was observed, microbial cells were attached to areas where there is roughage and pitting as was observed in Figure 10. Microbial cells seemed to show an increase in the number of cells on the surface of the substrate from day 2. However, it cannot be deduced whether there was a change in the composition of the cells on the same substrate because observations were made on different microplastic samples of the same polymer type on different days. Premature biofilm formation possibly develops between day 7 and day 14 in the river microcosms (Figure 11). In the LD-PE sample isolated on day 14, shearing and deterioration of the surface was observed, and clustering of cells preferred to form biofilms around these spots (Figure 11; day 14 image).

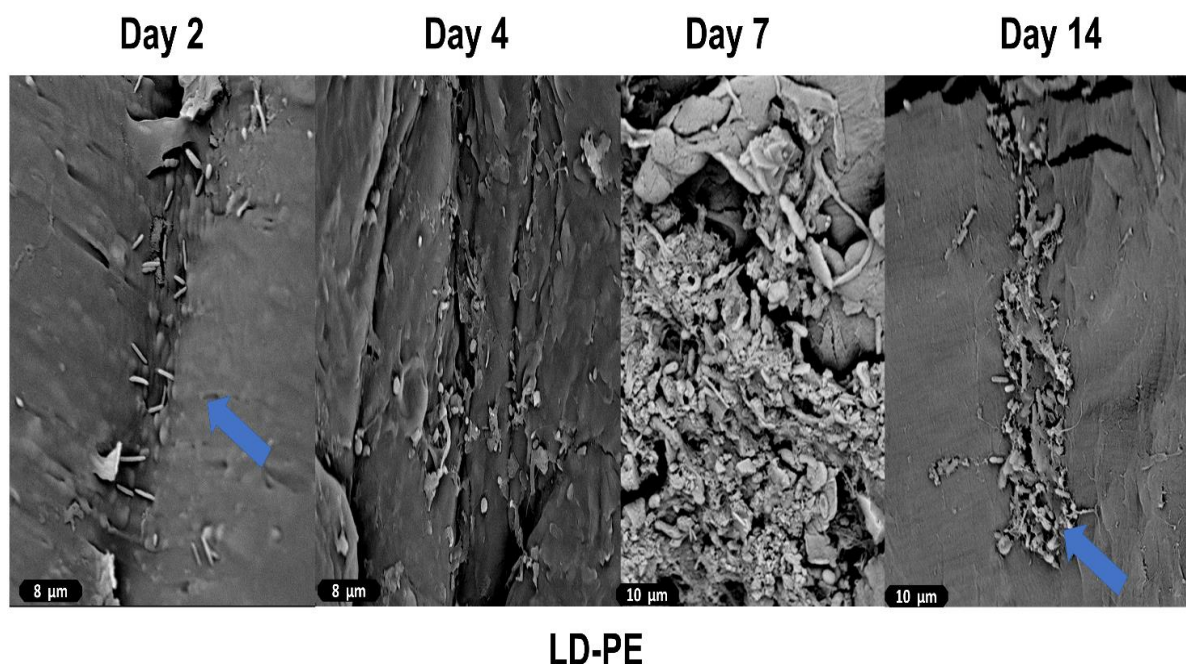


Figure 11: LD-PE (Low-density polyethylene) samples isolated from the river microcosms showing microbial colonization on the surface observed from day 2 to day 14.

The wood surface showed a higher abundance of microbial cells (Figure 12) than those observed on microplastic surfaces (Figures 10-11). The image from day 2 is not present due to a sample preparation error during the SEM coating step. On the image for day 4, it was observed that microbial cells were greater, and they covered the whole wood surface of the substrate (Figure 12). Microbial cells grouped themselves in the layered and folded parts of the wood substrates. Clusters of cells were visible and observed from as early as day 4, and microbial cell attachment on the surface increased with longer incubation periods on wood fragments. The SEM images revealed that microbial colonization on microplastics and wood substrates occurs rapidly upon introduction in the medium. The longer the substrates remained in the water, the more free-floating cells attached to their surface and built upon the cells that had initially attached. The results also indicated that cell attachment occurred at spots where there are crevices, pits, and grooves on the substrate surface.

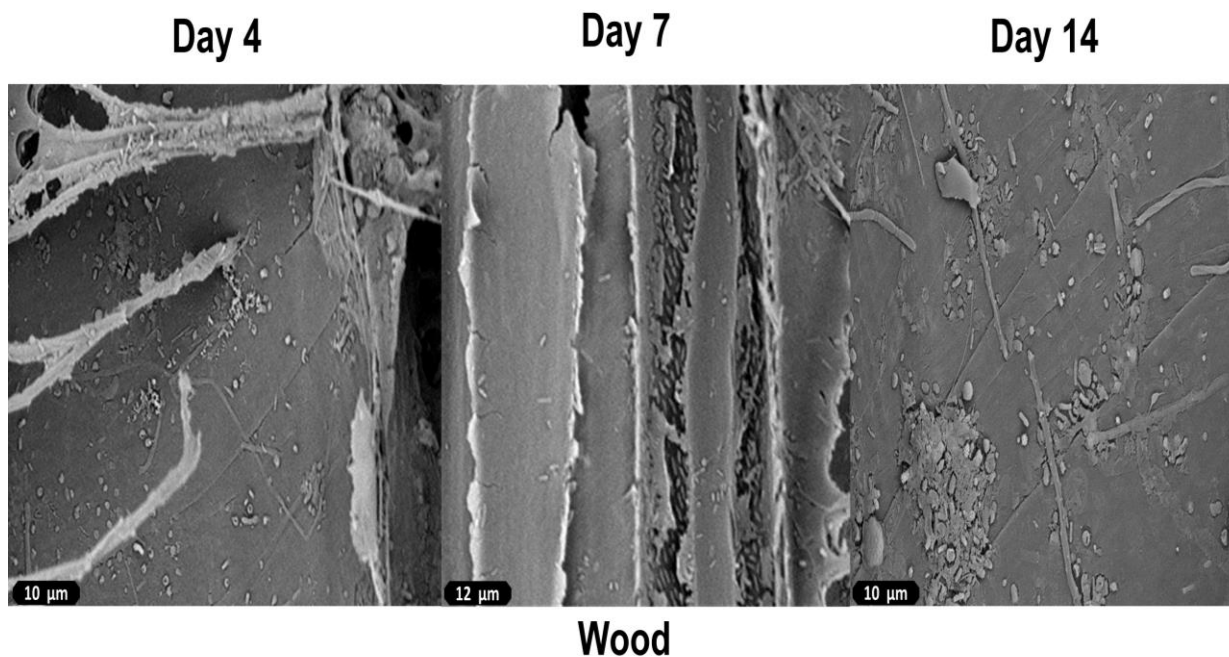
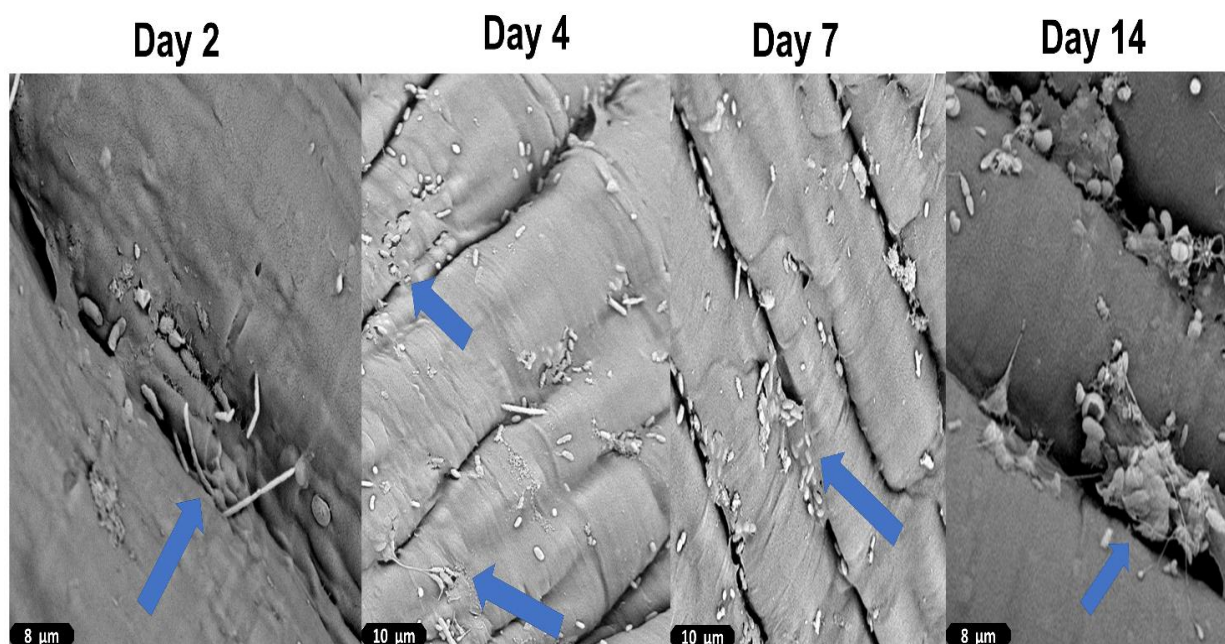


Figure 12: SEM images of wood substrates isolated from the river microcosms showing microbial colonization on the surface observed from days 4, 7, and 14.

4.3 Microbial colonization on WWTe microcosm substrates

The substrates were also incubated in WWTe to observe the microbial colonization in nutrient-rich settings. On the PP substrates on day 2, it was observed that microbial cells were attached to the creviced areas on the polymer surface (Figure 13). The cell abundance increased with a longer incubation period. Additionally, the PP sample isolated from the WWTe microcosm showed clusters of cells with a film covering on day 4, with some of the cell extensions stretched to other areas of the clusters. On day 7, it was observed that premature biofilms began to form at the folded parts of the microplastic surface supported by the film coating layer. On day 14, microbial biofilm formation was detected on the surface of the PP substrate. Microbial cells of spherical and rod-shaped cells were observed on the forming biofilm clusters.



PP

Figure 13: SEM images of polypropylene (PP) isolated from the WWTe microcosm showing microbial colonization on the surface observed from days 2, 4, 7, and 14.

The LD-PE microplastic sample isolated on day 2 had more cells attached to its surface (Figure 14) than the PP sample isolated on day 2 (Figure 13). It was observed that the rougher the surface, the more microbial cells attach to the surface. Some cells were found on the smooth part of the surface. Cell clusters potentially forming biofilms were observed in the early days of sampling on LD-PE (Figure 14). Different morphological structures of rods, spiral, spherical cells were consistently present on microplastic surfaces (indicated by yellow circles). On day 4 and day 7, premature but stable to surface biofilms were seen developing. Notably on day 7, microbial cells were attached to the degrading part of the microplastic. On day 14, a premature biofilm formation was observed. Furthermore, the microbial cell attachment on the surface of the substrate of the day 14 image was greater and dominated by rod cells.

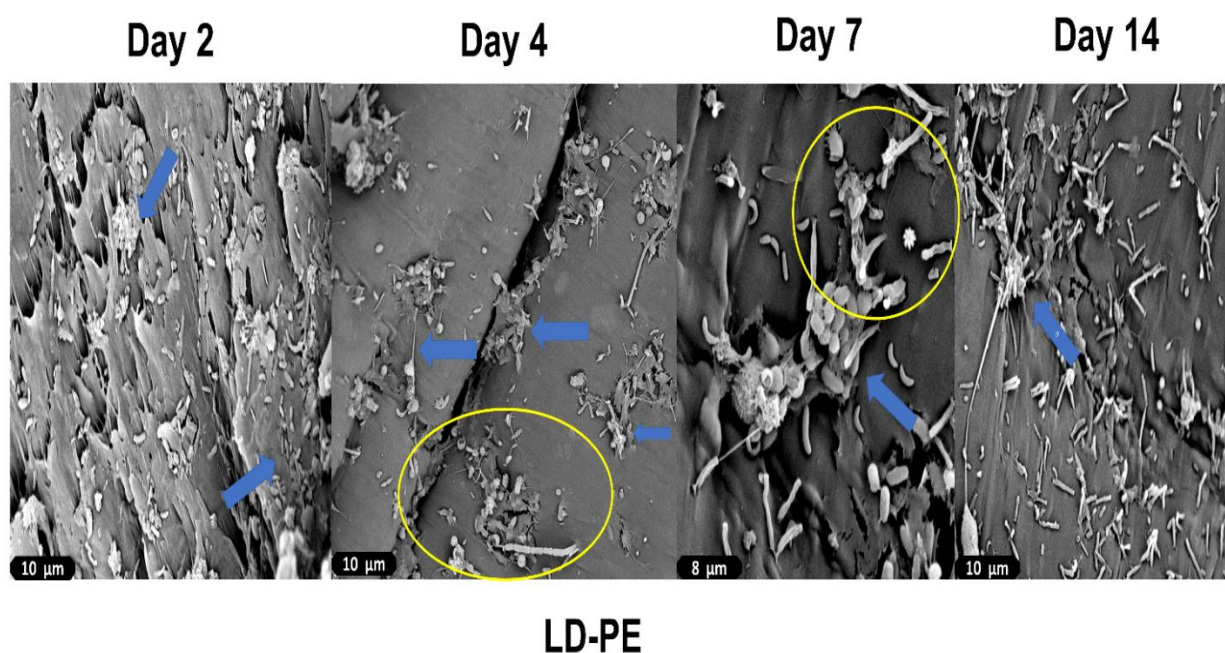
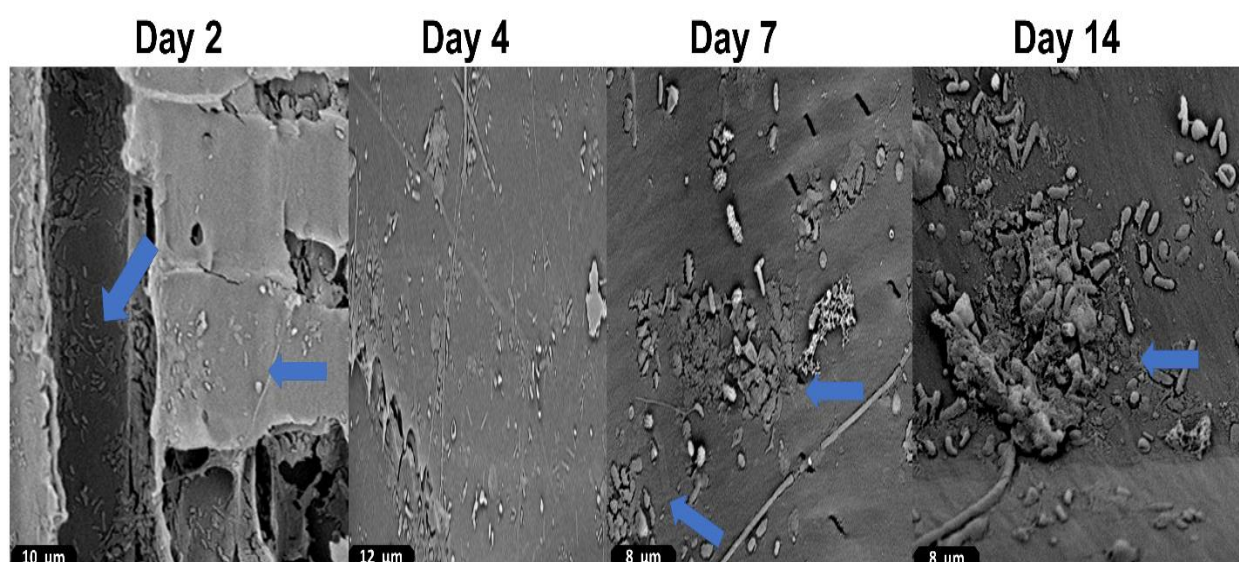


Figure 14: SEM images of LD-PE (low-density polyethylene) isolated from the WWTe microcosm. Microplastic samples were isolated on days 2, 4, 7, and 14 from the vessel.

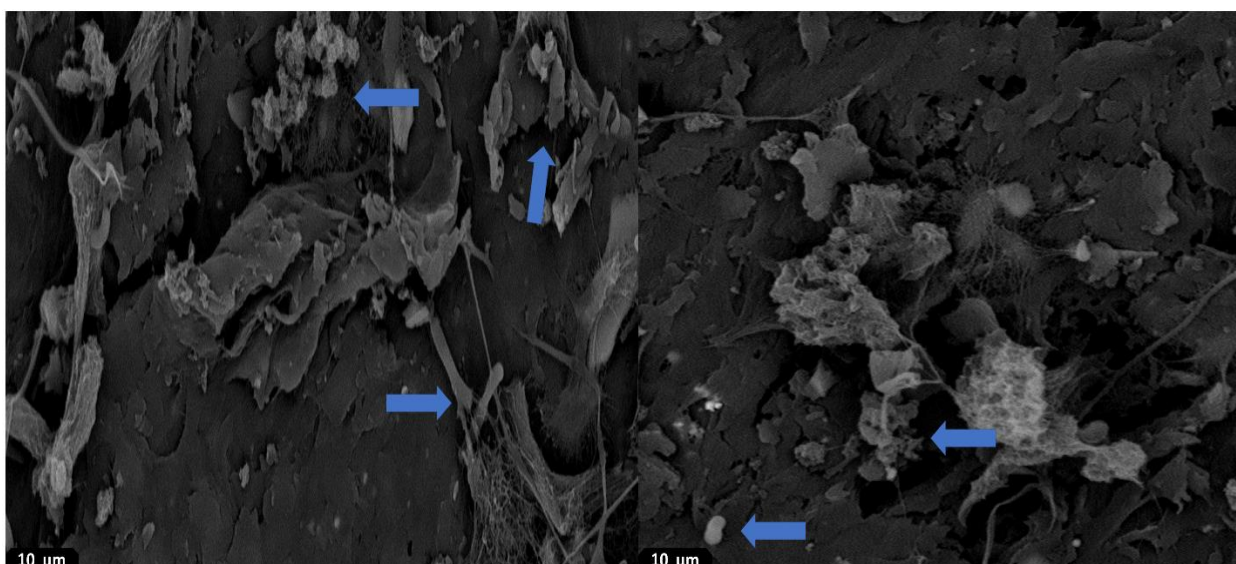
Due to wood substrate structure and compositional nature, it was observed that more bacterial cells were present in most of the wood substrates. This is due to the influences of the folded nature of the wood fragments used in this study (Figure 15). It can be noticed that microbial cells attached across the whole surface of the wood substrate varied with what was observed on microplastics. Wood is characteristically not smooth, and this explains the distribution of the cells on the surface. Clustering was visible from the earliest day of inspection. Microbial cell abundance was greater on the wood substrate. This suggested that the deeper parts and compartments of the wood fragments may contain more microbial cells due to the folded layers found on wood samples.



Wood

Figure 15: Wood substrate SEM images, isolated from the WWTe microcosm. Wood samples were isolated on days 2, 4, 7, and 14 from the vessel. Scales of the SEM images are shown on the bottom of each image.

Due to the type of PS microplastics used, it was difficult to observe bacterial colonization and biofilm development process by SEM visualization. Figure 16 provides a visualization of the PS substrates with microbial cells attached from the WWTe microcosm. It can be observed that microbial cells penetrated within the spongy parts of the PS substrate and build up more clusters in combination with the structural surrounding of the substrate. PS foams had far more surface area than solid microplastics. The abundance of microbial cells, in this case, could not be fully concluded. Therefore, 16S metabarcoding can provide more insight. SEM imaging revealed that microbial colonization on microplastics and wood fragments can occur within a few hours, and it is rapid in WWTP settings. This spans from the observation made on the substrates (microplastics and wood fragments) that were incubated in WWTe microcosm, which were more extensively colonized by microbial cells than those in river water. Filamentous and spherical cells occurred in rows and patches on the surface of the substrates. The microbial cells were mostly clustered where there are pits and grooves on the surfaces of the substrates. From day 4 to day 7, there was an increase in microbial cells on the surfaces of the substrates. Biofilms were observed on isolated microplastics on day 7. Wood substrates visibly showed more bacterial cells attached to the surface followed by LD-PE, PP, and PS.



Polystyrene (PS)

Figure 16: SEM images of PS (polystyrene) incubated in WWTe. Scales of the SEM images are shown on the bottom of each image.

4.4 DNA yield quantity and quality

The DNA concentration of the samples varied between sample types (substrates versus water). The DNA concentrations of the microplastic samples from the microcosm experiments that were viable for downstream application ranged from 0.70 to 6.10 ng/μl with a mean average of 3.58 ± 1.70 ng/μl. The concentrations of the wood samples ranged from 0.50 to 42.20 ng/μl with a mean average of 10.65 ± 17.69 ng/μl. The filtrate water samples from microcosm's samples ranged between 9.45 to 9.90 ng/μl with a mean average of 9.68 ng/μl. The DNA isolated from microplastics samples from the environment showed concentrations ranging from 0.40 ng/μl to 121.10 ng/μl with a mean average of 41.04 ± 41.88 ng/μl. The filtrate water samples' DNA concentrations ranged from 0.60 to 267.60 ng/μl with a mean average of 65.38 ± 101.02 ng/μl (Figure 17). The DNA quality was generally low for the microplastics and wood substrates used in the microcosm experiments with an absorbance ratio ($A_{260/280}$) of 0.82 to 1.68 and water samples at 1.88 to 1.95. Despite the overall low range of the extracted DNA as required, the microplastics isolated from the environment showed a better quality ranging between 0.77 to 1.87 than the microcosm samples. The DNA quality of the surrounding water samples ranged from 0.94 to 1.87. However, the DNA extractions led to the successful downstream application to yield the results that are presented in this study.

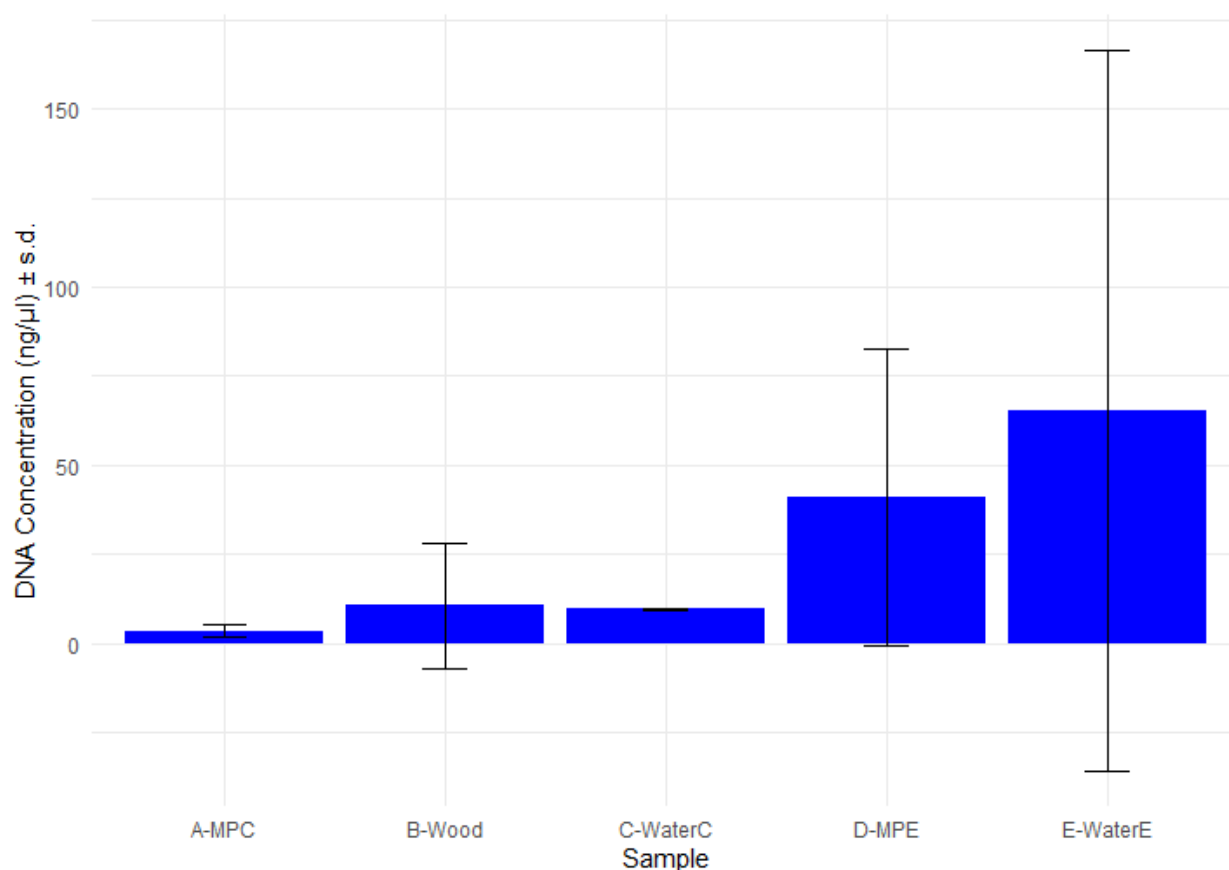


Figure 17: Mean average of DNA concentrations (ng/μl) $\pm$ standard deviation (s.d.) of the sample types: MPC (Microplastics from the microcosms); WaterC (Filtrate water from the microcosms); MPE (Microplastics from the environment); WaterE (Filtrate water from direct environmental sampling).

4.5 Bacterial community composition from microcosm samples

4.5.1 Characterization of the bacterial community composition from sample type and sample source

The BCC amongst microplastics and wood indicated that the dominant phyla were Proteobacteria and Bacteroidota. However, the composition varied throughout the incubation period of the microcosm experiments on the substrates. In the river microcosm (Figure 18), the top phyla of the total bacterial community from microplastic samples (F3) isolated on day 3 were Proteobacteria (52.67%) and Bacteroidota (46.88%). This was followed by phyla which were $\leq 1\%$. These are, in descending order Cyanobacteria, Campilobacterota, Actinobacteriota, Patescibacteria and Acidobacteriota. From microplastics sample C3 isolated on day 7, the phyla distribution is as follows, Proteobacteria (53.79%), Bacteroidota (40.77%), Verrucomicrobiota (4.42%), and Hydrodegenedentes (1.02%). Samples from day 14 (F14 and

F15) were dominated by Proteobacteria (75.29%), Bacteroidota (18.55%), Firmicutes (1.36%), Verrucomicrobiota, and Planctomycetota (1.21%). Hydrogenedentes, Chloroflexi, Acidobacteriota, Actinobacteriota, Bdellovibrionota, Myxococcota, Cyanobacteria represented $\leq 1\%$ of the phyla from these microplastics samples.

Wood substrates recovered from the river microcosm on day 3 from sample F4 were dominated by the following phyla: Proteobacteria (70.60%), Actinobacteriota (14.58%), Patescibacteria (4.85%), Bacteroidota (3.91%), Firmicutes (3.49%), and $\leq 1\%$ of the phyla were Deinococcota, Planctomycetota, Acidobacteriota, Verrucomicrobiota, Bdellovibrionota, Not_Assigned, Chloroflexi, and Cyanobacteria. In Sample D4 from day 7, the relative percentage abundance of Phylum Proteobacteria abundance was 91.09%, followed by Bacteroidota (7.61%). Firmicutes, Cyanobacteria, Verrucomicrobiota, Deinococcota and Campilobacterota were $\leq 1\%$. On Day 14, wood sample F14 showed a decrease in bacterial dominance of phylum Proteobacteria (36.26%). It was followed by phylum Chloroflexi (31.14%), Bacteroidota (14.53%), Acidobacteriota (5.39%), Planctomycetota (4.01%). Dependientiae, Verrucomicrobiota, Cyanobacteria, Firmicutes, Myxococcota, Actinobacteriota, Bdellovibrionota, and Patescibacteria made up $< 1\%$ of the total bacterial community on the substrate. The pooled water samples' (sample FF16) bacterial composition consisted of 45.68% of Proteobacteria, Bacteroidota (21.82%), Cyanobacteria (13.09%), followed by a rare proportion of phyla which were $< 5\%$ which consisted of Actinobacteriota, Verrucomicrobiota, Acidobacteriota, and Chloroflexi. The results indicated that microbial composition amongst the substrates differed from the filtrate water (FF16) sample from day 3 to day 14.

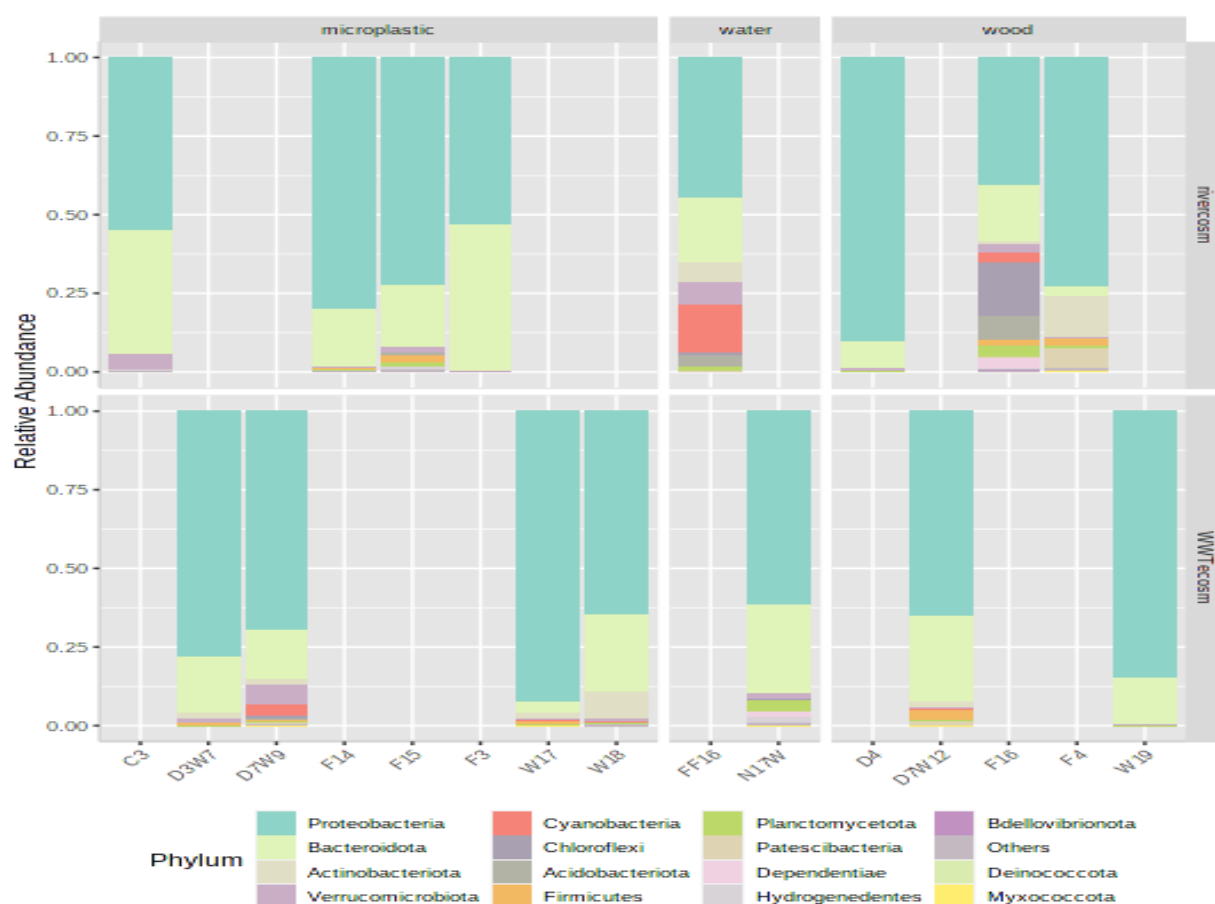


Figure 18: Relative abundance of the top 16 most abundant phyla based on 16S rRNA gene sequencing for bacterial assemblages from microplastics, wood, and the planktonic community in the river water and WWTe microcosms.

The results from the WWTe microcosm vessel indicate that Proteobacteria, Bacteroidota were the most dominant phyla in all samples (Figure 18). However, there was a fluctuation of bacterial composition in microplastic samples from day 3 (sample D3W7). The phyla distribution as observed in Figure 18 is as follows: Proteobacteria (76.17%), Bacteroidota (20.43%), followed by Actinobacteriota, Firmicutes, and Verrucomicrobiota, which represented < 1% of the bacterial phyla. On day 7 (Sample D7W9), there was a decrease in abundance of phylum Proteobacteria (64.34%) and phylum Bacteroidota (18.86%), with the presence of the following phyla which were < 5% Verrucomicrobiota, Cyanobacteria, Acidobacteriota, Actinobacteriota, Chloroflexi, Firmicutes, and Planctomycetota. After day 14, the microbial composition had shifted with Proteobacteria, Bacteroidota remaining dominant, followed by Actinobacteriota, Cyanobacteria, and Firmicutes. The filtrate (sample N17WW) of the WWTe had 16/17 of the top phyla present, with Proteobacteria (57.83%), Bacteroidota (29.91%), and Planctomycetota being the most top 3 abundant phyla. Phylum Deinococcota was not present post-incubation.

4.5.2 Bacterial community profiling and comparison analysis

4.5.2.1 Alpha diversity based on the sample type and sample source

Alpha diversity and significance test 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) as well as the source type of the microcosm (River versus WWTe). The results (Figure 19) revealed no significant difference between sample type (A: Chao1: $F = 3.493$, $p\text{-value} = 0.056$; B: Shannon index: $F = 0.098$, $p\text{-value} = 2.716$; C: Simpson index: $F = 0.762$, $p\text{-value} = 0.483$). No significant difference was observed between the river microcosm and WWTe microcosm when applying the T-test (D: Chao1: $p\text{-value} = 0.168$, statistic = -1.472; E: Shannon index: $p\text{-value} = 0.784$, statistic = -0.279 and C: Simpson index: $p\text{-value} = 0.517$, statistic = 0.678). Based on the featured OTUs for BCC from microplastics, wood, and water samples (Figure 19: A-C), filtrate water samples were richer, diverse, even, and dominant than the BCC from wood and microplastics substrates, respectfully. Figure 19 indicates that the BCC in the WWTe microcosm was richer and diverse, even, but less dominant than the BCC from the river microcosm (D-F). Microplastics were less rich and diverse than wood and surrounding water.

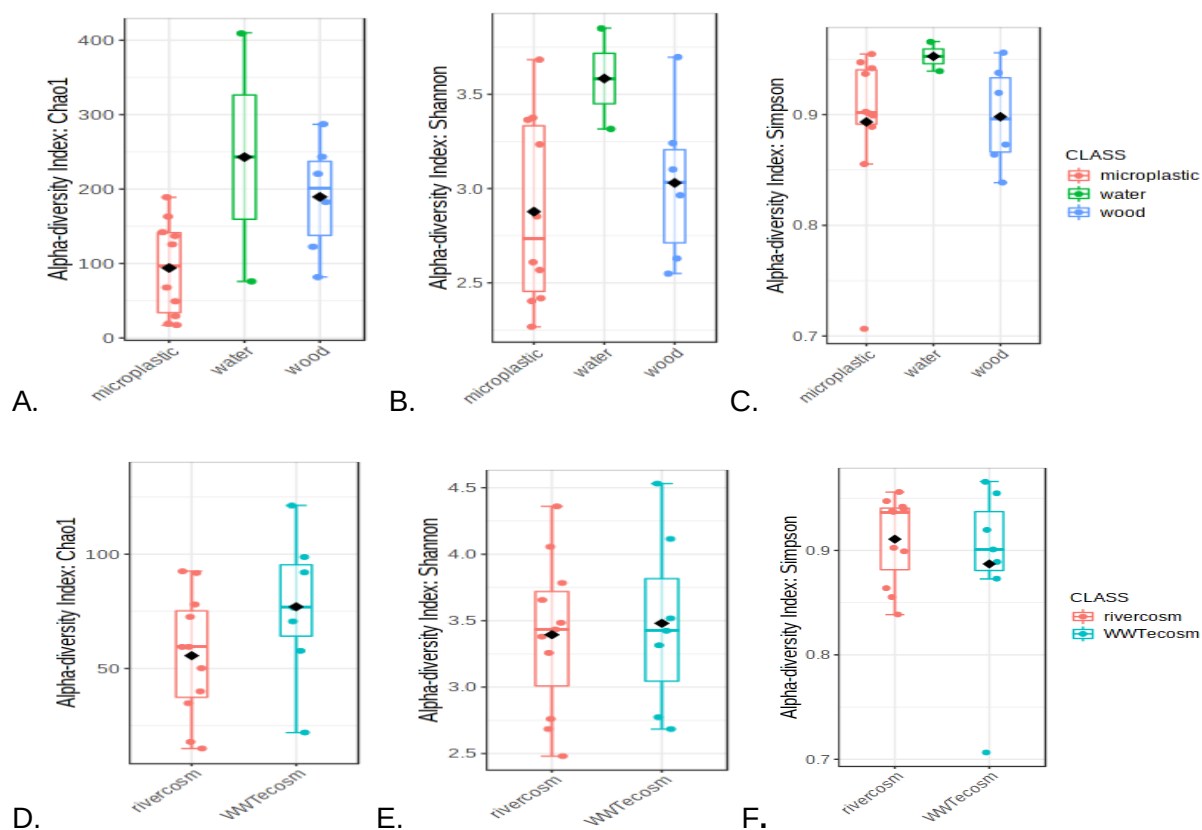


Figure 19: Alpha diversity indices (Chao1: A and D; Shannon: B and E; Simpson: C and F) for sample type (microplastics, wood, and water), including the diversity indices for the source type (river and WWTe) from microcosm experiments.

4.5.2.2 Alpha diversity of different substrates (PP, LD-PE, PS, and Wood)

Alpha diversity for substrate type was calculated at genus level. The bacterial community on wood substrates was richer and more diverse than LD-PE, PP, and PS bacterial composition. In terms of polymer type, it was observed that bacterial community distribution on the PP substrates was richer, more diverse, even, but less dominant than LD-PE and PS bacterial composition. The results revealed no significant difference between substrate type (A: Chao1: $F = 1.252$, $p\text{-value} = 0.337$; B: Shannon index: $F = 0.600$, $p\text{-value} = 0.669$; C: Simpson index: $F = 0.601$, $p\text{-value} = 0.669$). It was observed that the bacterial dominance was greater in LD-PE, followed by wood substrates, PS, and lastly PP (Figure 20: C). Considering all the substrates types, the results demonstrated that wood fragments present greater risks due to the increased BCC found on the substrates. However, the half-life of the wood fragments is shorter than that of the microplastics.

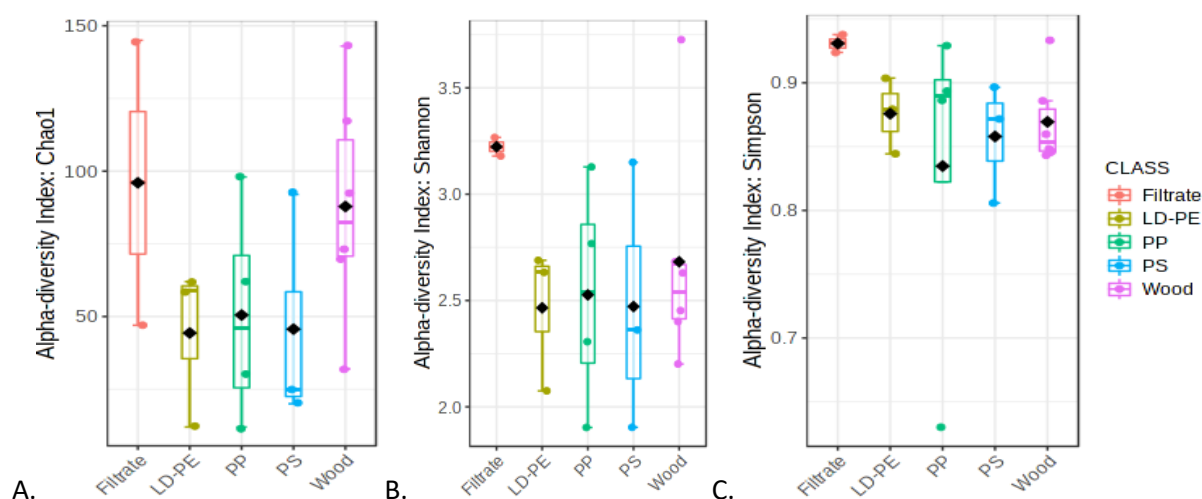


Figure 20: Alpha diversity indices for Chao1: A, Shannon: B, and Simpson: C of different substrates. LD-PE (Low-density polyethylene), PP (Polypropylene), and PS (Polystyrene).

4.5.3 Bacterial community membership

4.5.3.1 Distribution of bacterial community on sample types

The Linear discriminant analysis (LDA) effect size (LEfSe) was applied to compare the community abundance between the three sample types (microplastics, water, and wood) (Figure 21). The biomarker analysis showed that some families were significantly dominant between the sample types. For example, the *Alteromonadaceae*, *Aeromonadaceae*, and *Lactobacillaceae* families were dominant on microplastics. The wood samples were dominated (but not limited to) by the families *Moraxellaceae*, *Rhodobacteraceae*, and *Pseudomonadaceae*. The water samples showed dominance from the families *Flavobacteriaceae*, *Phormidiaceae*, and *Microscillaceae* (Figure 21). The bar graph only indicates the dominance taxa based on one sample type in comparison to the others. Thus, the LEfSe biomarkers were further looked at in detail in table format to observe if there were biomarker groups that would be solely present in one group when compared to the other sample types (Table 3-4). This was performed at the genus level for both river water and WWTe microcosms.

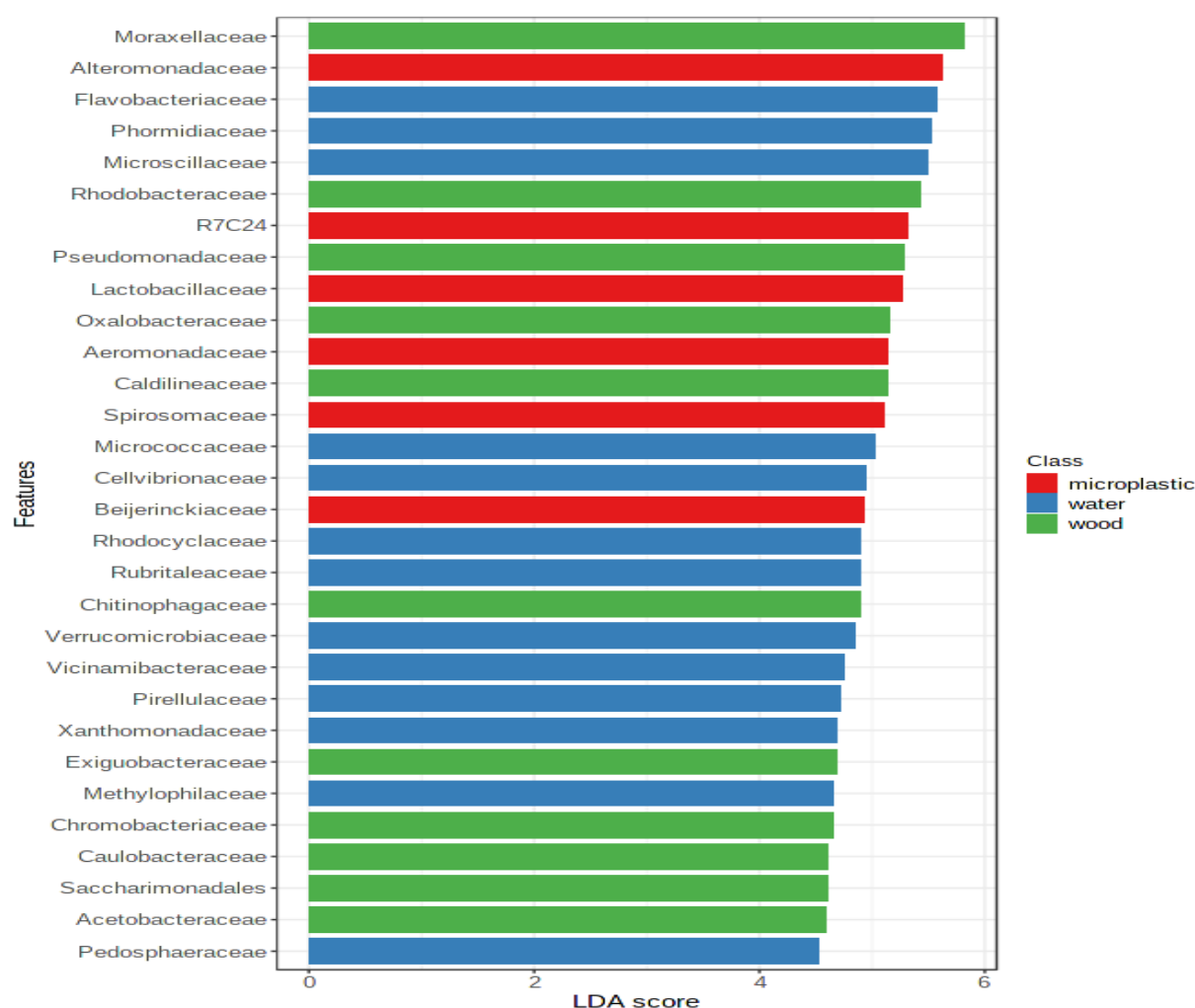


Figure 21: Taxonomic bar graph showing overall community membership dominance at family level generated using LEfSe analysis for microcosm sample types (microplastics, water, and wood).

Table 3 shows some of the genera known to contain opportunistic pathogens that were common in both river and WWTe microcosm samples. The results from the river water microcosms show that genera *Aeromonas*, *Acinetobacter*, *Roseomonas*, and *Zoogloea* were detected and dominant in microplastics and wood substrates but not in filtrate river water samples. The results further indicated that genera *Aeromonas* and *Acinetobacter* were more enriched on microplastics than wood samples. *Roseomonas* and *Zoogloea* were more dominant on wood than microplastic substrates in the river water microcosms. Genus *Pseudomonas* was found to be greater in wood substrates in both river and WWTe microcosms. This genus was the least dominant in microplastics from the river water microcosm. However, it was dominant in wood and microplastics, and lastly, the filtrate WWTe samples from the WWTe microcosm.

Table 3: Tabulation of community membership at genus level's distribution on microplastics and wood substrates versus filtrate water samples.

Genus					
	<i>Aeromonas</i>	<i>Acinetobacter</i>	<i>Pseudomonas</i>	<i>Roseomonas</i>	<i>Zoogloea</i>
p-value	0.469	0.347	0.927	0.138	0.589
Microplastics	436990	904190	257980	3700.3	13010
River water	0	0	272730	0	0
Wood	210580	174220	698840	136060	49657
LDA score	5.34	5.66	5.34	4.83	4.39
p-value	0.498	0.299	0.299	0.053	0.385
Microplastics	81780	213600	390490	0	15293
WWTe	16056	87541	170760	7478.3	0
Wood	157080	1956500	428520	2296	10016
LDA score	4.85	5.97	5.11	3.57	3.88

Abbreviations: LDA = Linear Discriminant Analysis; WWTe = Wastewater treatment effluent

LEfSe biomarker analysis tables of results highlighting all distribution of different genera found on microplastics, wood, and water samples are shown in the Annexures section as Table S1-A (river microcosm) and Table S1-B (WWTe). The results revealed that certain genera from river water microcosm vessels were present on microplastics and wood substrates but not detected in filtrate water samples. The results further showed that most of the genera would be present on all three sample types and/or be present on WWTe water samples and wood substrates but not microplastics. The microplastics and wood substrates from the WWTe microcosm were more enriched with potentially pathogenic genera such as *Coxiella*, *Clostridium*, *Exiguobacterium*, and *Mycobacterium* than in water samples.

4.5.3.2 Distribution of bacterial community amongst the substrate type

The distribution of genera data on substrate type was also evaluated using LEfSe biomarker analysis. Table 4 shows selected genera with clinical importance. Furthermore, some of the species in these genera have been reported to have properties that enable them to degrade different types of polymers. The results in Table 4 show that *Aeromonas* and *Acinetobacter* were prevalent in microplastics and wood substrates and greatly enriched PS, PP, and LD-PE polymers respectively. However, they were not detected on filtrate water samples from the river water microcosm vessel. Genus *Pseudomonas* was prevalent in all substrate types, including the filtrate water from the river and WWTe microcosms. However, in terms of polymers, it was greatly enriched on PS, followed by PP and LD-PE substrates. Other genera such as *Ideonella* and *Exiguobacterium* were only present in the WWTe, it was also observed

that genus *Ideonella* and *Shewanella* were present on PS and not on PP and LD-PE microplastic substrates (Table 4 and Annexure Table S1-C and S1-D).

Table 4: The distribution of genera on LP-PE, PP, PS, and wood substrates in the river and WWTe microcosm.

Genus	p-value	Filtrate Water	LD-PE	PP	PS	Wood	LDA score	
<i>Aeromonas</i>	0.563	0	317140	955680	38145	210580	5.68	
<i>Acinetobacter</i>	0.672	0	495190	506630	1710700	174220	5.93	River
<i>Pseudomonas</i>	0.883	272730	205040	256530	312360	698840	5.39	
<i>Aeromonas</i>	0.607	15130	6416.5	531000	118150	157560	5.42	
<i>Acinetobacter</i>	0.587	82492	53129	471290	1411900	1948900	5.98	WWTe
<i>Pseudomonas</i>	0.636	160910	166320	259250	389650	427530	5.12	
<i>Ideonella</i>	0.319	9534.3	0	0	161230	687.1	4.91	

Abbreviations: LDA = Linear Discriminant Analysis; LD-PE = Low-density polyethylene); PP = Polypropylene; PS = Polystyrene; WWTe = Wastewater treatment effluent.

4.6 Microplastics isolated from surface water

Throughout the sampling period of September to November 2020, a total of 206 putative microplastics were identified from approximately 1800 litres of filtered surface water across 3 sampling stages in five sites. Microplastics were visually identified and removed from the debris using sterile forceps and grouped according to their classification type (fibres, fragments, films, foams, and microbeads) (Figure 22). The particles that were > 5 mm were not considered for analysis for this study as they were over the size class definition of microplastics, which is < 5 mm (Bouwman *et al.*, 2018). Based on observation during sampling, only Site G3 had various microplastic types such as fibres, fragments, films, foams, and microbeads. Sites M1, O2, W4, and T5 (Figure 8) were dominated by microplastic fibres throughout the sampling stages. The following microplastic types were identified under a stereomicroscope at a 40X magnification.



Figure 22: Representative putative microplastics from environmental samples photographed under a stereomicroscope after filtration of surface water in the Mooi River in NWP.

Fibre particles made up 77.2% of the microplastic samples and were found in all the sampling sites (Figure 20). These were filamentous string-like shaped polymer pieces with a variety of colours (blue, red, pink, and purple) as shown in Figure 22. The fragments collected made up 9.7% of the total microplastics collected. These were pieces with jagged edges and solid textures with a three-dimensional shape. Some fell in part with microbeads (2.9%) which were round shaped with chipped edges. Foams included common polystyrene (PS) sponge-like texture with various irregular shapes which were spherical, flat, and made up 7.3% of the microplastics. Pieces classified as films made up 2.9% and were flat thin pieces with irregular shapes.

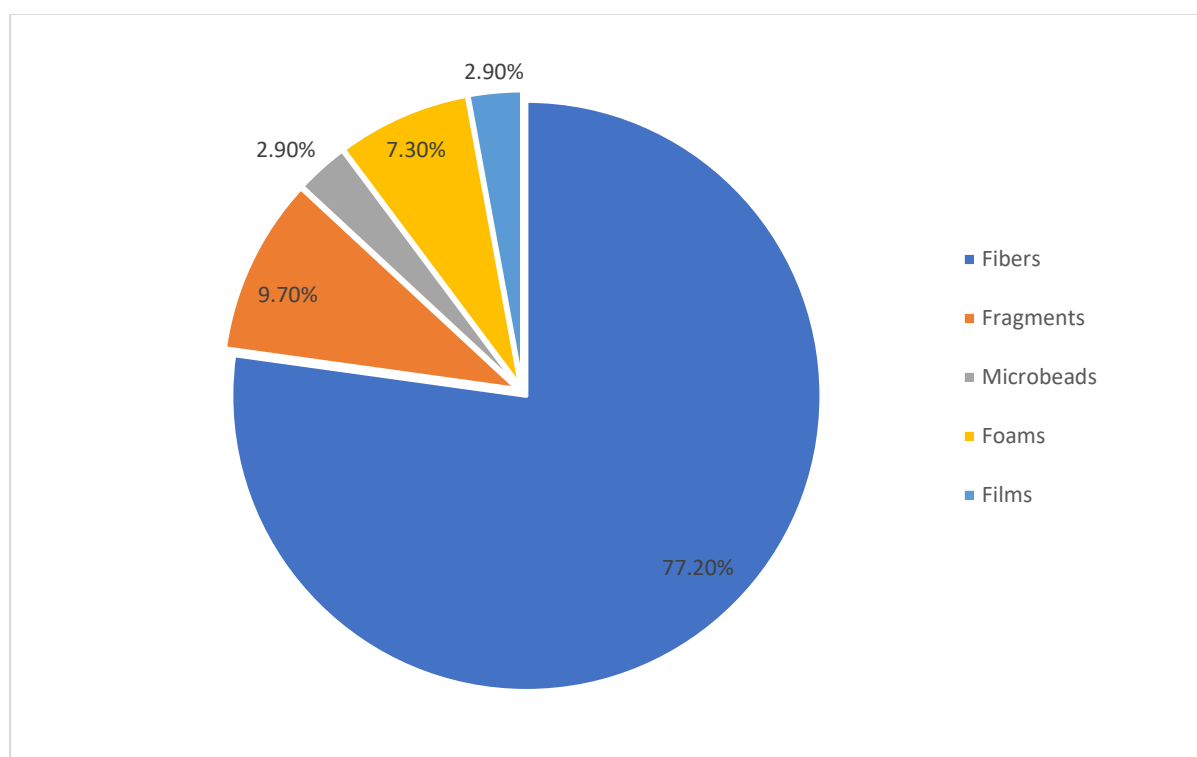


Figure 23: The prevalence of collected microplastics types from five sampling sites in the Mooi River catchment.

4.7 Microbial characterization on environmental microplastics

Some microplastics isolated from surface running water during the field sample collection in 2020 were selected and subjected to the viewing of the surface by SEM. Figure 24 shows images of the surfaces of the microplastics. The first group (Figure 24: group A) is the stereomicroscopic view of the samples before being subjected to chemical dehydration steps for SEM. It was observed a layer of inorganic matter covered the microplastics. Following the dehydration steps (which allowed for the fixation of cells onto the surface), the samples were viewed using the Phenom SEM system. It was observed that the surfaces of the microplastics were densely colonized by eukaryotic and bacterial cells (Figure 21: group B). The microplastic

surfaces had some pitting and creviced surfaces, which indicates a form of degradation. Furthermore, it was observed that the areas that were rough and creviced were heavily colonized and covered with biofilms, unlike the smoother part of the surface.

The view at 10 μm (Figure 21: group C) showed the presence of cocci, streptococci, and rod-like filamentous bacterial cell shapes on the surface of microplastics (Blue arrows). The SEM images also showed a presence of diatoms of different morphology attached to the microplastics (yellow arrows). Biofilms were observed on the microplastic surfaces (blue circle), based on the protective coating formed by bacterial cells. Although not clear enough, some cocci cells can be observed within the covering of the layer of biofilms. The images showed some dividing cells (red arrows), which could suggest the active growth of the cells. DNA isolation and 16S metabarcoding analysis from microplastic fragments led to the identification of the BCC on the surface of microplastics.

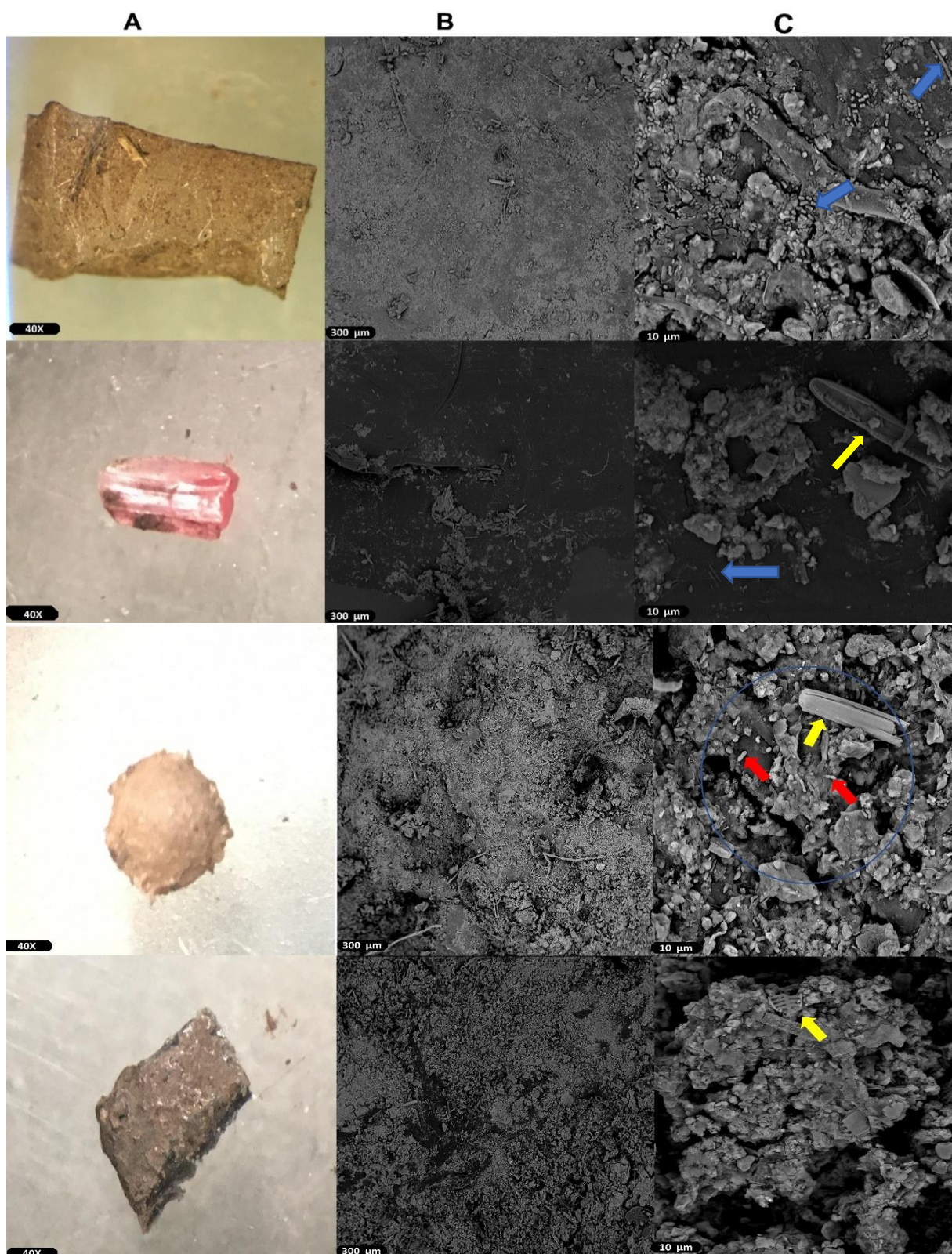


Figure 24: Stereomicroscope and SEM images showing examples of the surfaces of microplastic fragments covered with biofilms made of a diverse microbial community. (A) Stereomicroscope pictures of microplastic fragments; (B) The depiction of the representative surface of the microplastic showing the extent of coverage by microorganisms and (C) A 10 μm view of bacterial and diatomic cells attached on the surface of the microplastics.

4.7.1 Bacterial community composition from field microplastics and water

Both microplastics and corresponding filtrate water samples were dominated by the phylum Proteobacteria. Dominance and representation of phyla varied amongst microplastics and water at each site. The phyla distribution in microplastics from site M1 (M1P) was as follows: Proteobacteria (53%), Bacteroidota (20%), Actinobacteriota (14%), Cyanobacteria, and Verrucomicrobiota with a representation of 5%. The water sample (M1W) phyla distribution was also dominated by Proteobacteria (75%), Bacteroidota (8%), followed by Firmicutes (8%), Actinobacteriota, Verrucomicrobiota. Site O2 microplastic samples (O2MP) were represented by Proteobacteria, which showed a representation of 75% followed by Bacteroidota (16%), Planctomycetota, Cyanobacteria, Verrucomicrobiota, and Other showed < 1% representation. The phyla distribution in water (O2W) found in Site O2 was represented by Proteobacteria (58%), Bacteroidota (22%), Patescibacteria (9%), Actinobacteriota, Chloroflexi, Firmicutes, Bdellovibrionota, and Verrucomicrobiota (Figure 25).

The overall microplastics from Site G3 (G3MP) were markedly dominated by Proteobacteria, Patescibacteria, Bacteroidota, Planctomycetota, Campilobacterota, and Chloroflexi. Actinobacteriota, Firmicutes, Bdellovibrionota, Verrucomicrobiota, Other, and Myxococcota represented < 5% of the total bacterial community. The water samples (G3W) from Site G3 were dominated by the following phyla: Proteobacteria (58%), Bacteroidota (14%). They were followed by Planctomycetota, Acidobacteriota (5%), Chloroflexi (4%), Actinobacteriota (3%), Verrucomicrobiota, Patescibacteria, Cyanobacteria, Firmicutes, and Gemmatimonadota which $\leq 2\%$. Phyla distribution from microplastics from Site W4 was as follows: Proteobacteria (89%) followed by Bacteroidota, Planctomycetota, Firmicutes, Others, Verrucomicrobiota for microplastics sample W4MP. Filtrate water sample W4W was represented by 45% Bacteroidota, Actinobacteriota (23%), Proteobacteria (22%), Cyanobacteria, Firmicutes, Other, and Verrucomicrobiota. Microplastics sample (T5MP) were characterized by Planctomycetota, Firmicutes, Myxococcota phyla. Water samples contained 80% of the Proteobacteria phyla. A rare proportion of Campilobacterota, Firmicutes, Others made up < 1% of BCC. Proteobacteria and Bacteroidota $\geq 60\%$ dominated the water samples in Site T5, followed by Planctomycetota, Campilobacterota, and Others.



Figure 25: Relative abundance of the top 20 most abundant phyla based on 16S rRNA metabarcoding for taxonomic composition from microplastic (MP) and water (W) samples from each respective site.

4.7.2 Alpha diversity indices of field microplastic samples

The variable microplastic samples from Site G3 were pooled together to represent one microplastic group for this site for a fair comparison with other sites and/or samples. The data were normalized to the equal library size for rarefaction at over 97% goods' coverage to include all sites. The calculated results and alpha diversity graphs are presented in Figure 26. The results showed no major differences between species richness, diversity, evenness, and dominance calculated at the genus level. The microbial composition in water samples was slightly richer and diverse than in microplastics. In terms of species evenness and dominance, microplastics samples showed a slightly higher Simpson value (0.924) than in water samples (0.921). No significant differences were observed between microplastics and water using the

T-test. Chao1: Statistic = -0.125, p-value = 0.904; Shannon: Statistic = -0.007, p-value = 0.946 and Simpson: Statistic = 0.061, p-value = 0.953.

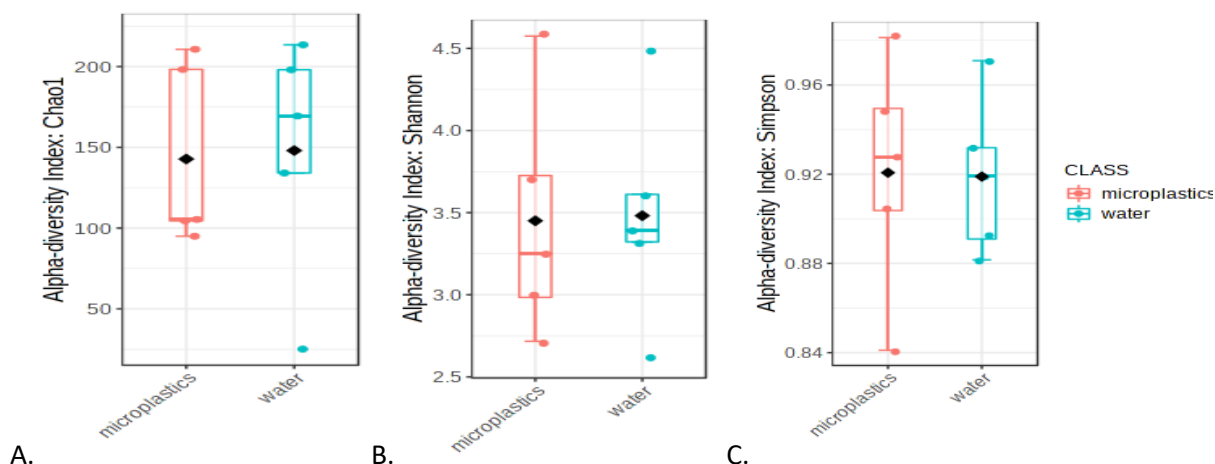


Figure 26: Bacterial alpha diversity indices (A-Chao1, B-Shannon, and C-Simpson) for sample type (microplastics and water).

4.7.3 Community membership distribution between field microplastics versus the surrounding water

The analysis of LEfSe biomarkers was conducted at family level (Figure 27 and Table 5) and genus level (Table 6) to quantitatively determine the distribution of bacterial communities between the field microplastics samples and the surrounding river water. Figure 27 shows a maximum number of dominant bacterial taxa at family level between microplastics and water samples. Water samples were dominated by (but not limited to) the following families *Moraxellaceae*, *Alteromonadaceae*, *Spirosomaceae*, *Microbacteriaceae*, *Exiguobacteriaceae*, and *Pseudomonadaceae*. The microplastics samples were dominated by *Diplorickettsiaceae*, *Peptostreptococcaceae*, *Enterobacteriaceae*, and *Acetobacteraceae*. Further analysis of the LEfSe biomarkers by numbers was done to observe in detail whether these members were present or absent on the different sample types (microplastics versus water), is shown in table format on Table 5 and 6. The results indicated that some bacterial taxa may be selectively enriched on microplastics than in water. Similar results in terms of other families being enriched more on water or microplastics were also observed in the microcosm experiments.

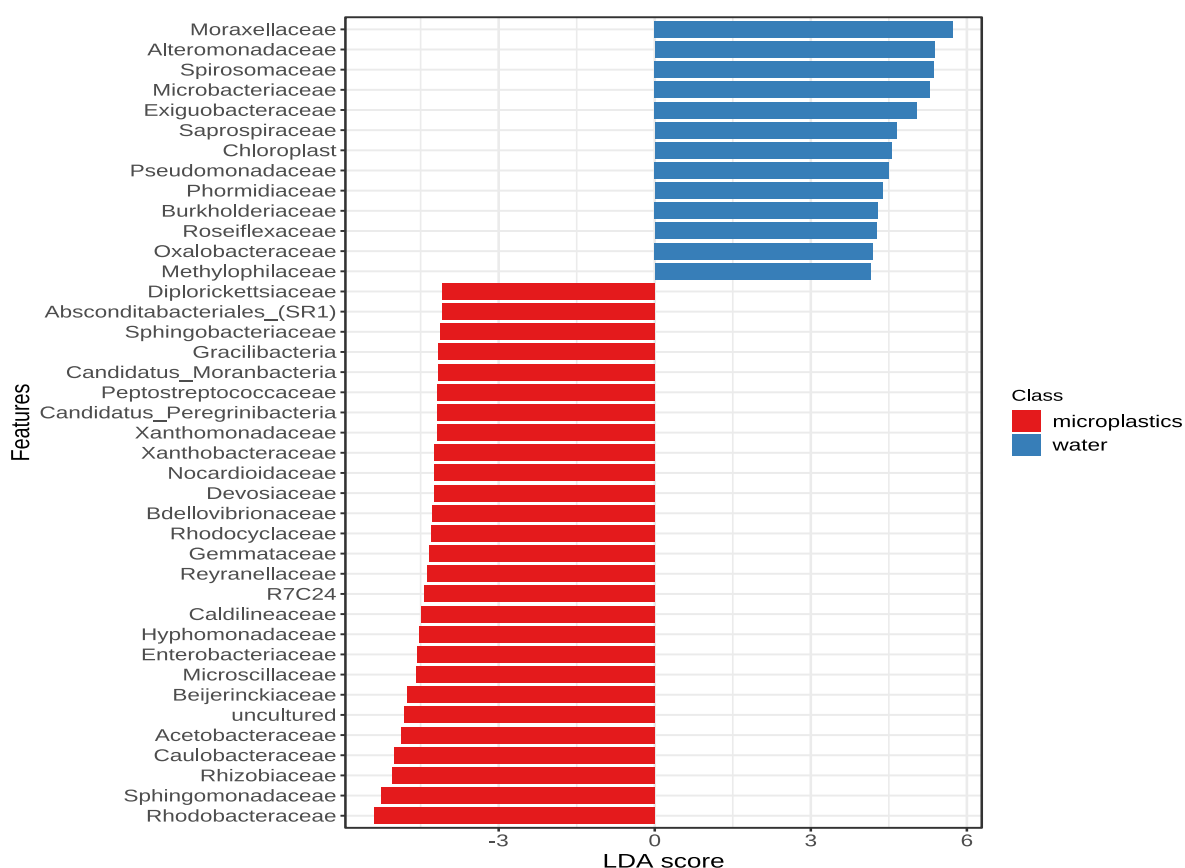


Figure 27: Taxonomic bar graph showing overall community membership dominance at family level between the microplastic and water samples from the environment (2020 sampling) generated using LfSe biomarker analysis.

Table 5 shows the top 11 discriminating features between the sample types. The results showed that some families were significantly higher (p -value < 0.050) on microplastics than the surrounding water community for example, *Enterobacteriaceae* (p -value = 0.007), *Isosphaeraceae* (p -value = 0.019), *Legionellaceae* (p -value = 0.019) were among the top 11 families that were dominant on microplastics. It was also determined that genera (*Escherichia*, *Shigella*, *Legionella*) belonging to the above families (*Enterobacteriaceae* and *Legionellaceae*) were significantly higher on microplastics than water (Table 6). The results also revealed that some groups were significantly higher in the surrounding water than microplastics samples. These included genus *Exiguobacterium* (p -value = 0.001) which belongs to the *Exiguobacteraceae* family (p -value = 0.016). The SEM images showed dominance and dense colonization of cocci and rod-like structured cells, many members identified by 16S metabarcoding show a dominance of cocci and rod shape groups. Lists of other groups showing core membership distribution on sample types (microplastics versus water) and source type (river and WWTe) are available in the Annexure section listed as Table S2-A and Table S2-B.

Table 5: Distribution of bacterial taxa (by numbers) at family level on microplastics and water samples.

Family	P-value	microplastics	water	LDA score
<i>Enterobacteriaceae</i>	0.007	81808	1012.6	-4.61
<i>Exiguobacteraceae</i>	0.016	3058.5	221850	5.04
<i>Isosphaeraceae</i>	0.019	5700.2	0	-3.46
<i>Legionellaceae</i>	0.019	7704.7	0	-3.59
<i>Geminicoccaceae</i>	0.019	2258.7	0	-3.05
<i>Tannerellaceae</i>	0.019	7479.3	0	-3.57
<i>Bacteroidaceae</i>	0.019	21712	0	-4.04
<i>Reyranellaceae</i>	0.034	41324	1635.9	-4.3
<i>Hydrogenedensaceae</i>	0.034	7620.3	262.23	-3.57
<i>Rhizobiaceae</i>	0.047	223250	35597	-4.97
<i>Alteromonadaceae</i>	0.047	121010	805690	5.53

Table 6: Distribution of top groups on microplastics and water at genus level determined using LEfSe analysis.

Genus	P-value	microplastics	water	LDA score
<i>Exiguobacterium</i>	0.001	2094.4	224420	5.05
<i>Legionella</i>	0.024	14697	0	-3.87
<i>Pedobacter</i>	0.034	30230	765.68	-4.17
<i>Escherichia_Shigella</i>	0.039	27291	0	-4.14
<i>Rheinheimera</i>	0.051	330670	798230	5.37
<i>Hirschia</i>	0.058	48758	2541.7	-4.36
<i>Gemmata</i>	0.061	13484	0	-3.83
<i>alpha_cluster</i>	0.061	3934.9	0	-3.29
<i>Bacteroides</i>	0.061	17497	0	-3.94
<i>Methyloversatilis</i>	0.061	2148.5	0	-3.03

Red numbers: Indicate a p-value < 0.050 which shows significance; (-) in Linear Discriminant Analysis (LDA) score indicates the skewness of distribution, the negative sign indicates prevalence on microplastics.

4.8 Detection of AmpC β -lactamase and *int1* genes

Microcosm and environmental microplastics with filtrate water samples were screened for seven resistance genes. Six of the genes are clinically relevant, namely the AmpC β -lactamase gene groups. The *int1* gene is a known gene cassette and is commonly detected in densely polluted areas (Gillings *et al.*, 2014). Figure 24 shows representatives of the genes that were screened for using end-point PCR.

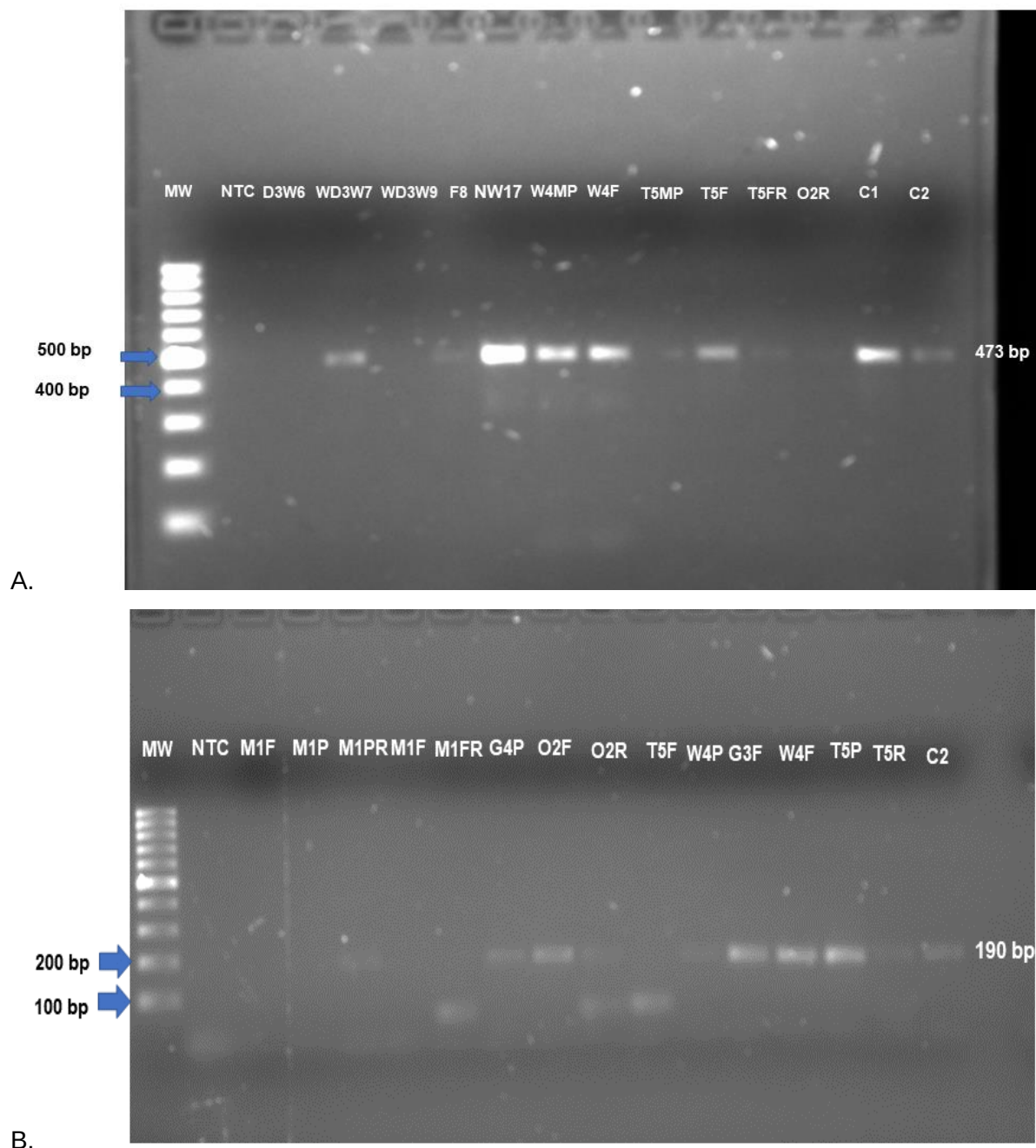


Figure 28: 1.8% (w/v) agarose gel electrophoresis showing examples of the genes that were screened using end-point PCR on different samples from field and microcosms analysis. MW: molecular weight marker of 100 bp (O'GeneRuler, Thermo Fischer Scientific, USA). NTC

(Non-template control), C (control). The approximate sizes of the bands are indicated on the sides of the gels. (A) Gel image for visualization of the *int1* gene, (B) Gel image for FOX gene group.

4.8.1 Detection of six AmpC β -lactamase genes and the *int1* gene from microcosm samples

Microplastics, wood, and water (river and WWTe) samples from 2019 microcosm studies were screened for six AmpC β -lactamase gene groups and the *int1* gene. The results were tabulated and presented in Table 7. From the microcosm experiments, the FOX gene group was detected in all samples (n= 15) from both the river water and WWTe microcosms. MOX/CMY gene group was detected in only 6/15 samples from the WWTe. DHA was found in 3/15 samples of wood and filtrate water samples and none in microplastics incubated in WWTe vessel. Overall, amongst the AmpC β -lactamases gene groups, FOX was the only gene group present in all substrates (microplastics and wood) and filtrate water samples from the river water microcosm. In the WWTe microcosm, only 3/6 AmpC gene groups (FOX, DHA, MOX/CMY) were detected. However, the DHA gene group was only found in wood substrates (WD7W12 and W19) and filtered WWTe water samples (NW17). The *int1* gene was detected in 5/15 samples of microplastics, wood, and filtrate water from the WWTe microcosms.

Table 7: The prevalence of the six AmpC β -lactamase gene groups and the *int11* gene from both river and WWTe microcosm samples.

Sample	Substrate	Source	ACC	DHA	FOX	LAT/CMY/BIL	MIR/ACT	MOX/CMY	<i>int11</i>
F3	MP	River	-	-	D	-	-	-	-
F4	Wood	River	-	-	D	-	-	-	-
F3C	MP	River	-	-	D	-	-	-	-
F4D	Wood	River	-	-	D	-	-	-	-
F14	MP	River	-	-	D	-	-	-	-
F15	MP	River	-	-	D	-	-	-	-
F16	Wood	River	-	-	D	-	-	-	-
FFIL16	Water	River	-	-	D	-	-	-	-
WD3W7	MP	WWTe	-	-	D	-	-	D	D
WD7W9	MP	WWTe	-	-	D	-	-	D	-
WD7W12	Wood	WWTe	-	D	D	-	-	D	D
W17	MP	WWTe	-	-	D	-	-	D	-
W18	MP	WWTe	-	-	D	-	-	D	D
W19	Wood	WWTe	-	D	D	-	-	-	D
NW17	Water	WWTe	-	D	D	-	-	D	D

D = detected; (-) = undetected; MP (microplastic); WWTe (Wastewater treatment effluent)

4.8.2 Detection of six AmpC β -lactamase genes and the *int1* gene the field environmental samples

Environmental samples of microplastics and surrounding water samples obtained in 2020 from the specific sites along the Mooi River indicated on the map in Figure 8 were also screened for the six AmpC β -lactamase gene groups and the *int1* gene. From Table 8, it can be observed that ACC and MIR/ACT gene groups were detected in both microplastic and water samples in all the sites. In Site M1, only 2/6 AmpC gene groups (ACC and MIR/ACT) were detected in microplastics and water samples. The ACC and MIR/ACT gene groups were detected in microplastic samples from Site O2. However, in filtrate water samples from Site O2, 5/6 AmpC gene groups (ACC, DHA, MOX/CMY, MIR/ACT, LAT/CMY/BIL) were present. In both microplastic and water samples from Site G3, 5/6 AmpC gene groups (ACC, FOX, MOX/CMY, MIR/ACT, LAT/CMY/BIL) were detected. In Site W4, 6/6 AmpC gene groups were detected in both microplastic and water samples. These are samples that were obtained from the WWTe flowing into the river system. In Site T5, 3/6 genes (ACC, DHA, MOX/CMY, and MIR/ACT) were found on microplastics. Conversely, 5/6 genes (ACC, DHA, FOX, MIR/ACT, LAT/CMY/BIL) were detected in water samples. The *int1* gene was detected in water samples from Sites O2, G3, W4, and T5 and only detected on microplastic samples in Sites G3 and W4.

Table 8: The distribution of the six AmpC β -lactamase gene groups and the *int1* gene from environmental microplastics and water obtained from various sites in the Mooi River.

Sample	Site	Source	ACC	DHA	FOX	LAT/CMY/BIL	MIR/ACT	MOX/CMY	<i>int1</i>
M1P	M1	MP	D	-	-	-	D	-	-
M1F		water	D	-	-	-	D	-	-
O2P	O2	MP	D	-	-	-	D	-	-
O2F		water	D	D	-	D	D	D	D
G3P	G3	MP	D	-	D	D	D	D	D
G3F		water	D	-	D	D	D	D	D
W4P	W4	MP-WWTe	D	D	D	D	D	D	D
W4F		WWTe	D	D	D	D	D	D	D
T5P	T5	MP	D	D	-	-	D	D	-
T5F		water	D	D	D	D	D	-	D

D = detected; (-) = undetected; MP (microplastic); WWTe (Wastewater treatment effluent)

4.9 Determining the quantity of ARG/16S rRNA on the BCC of microplastics versus water

Standard curves were generated for the 16S rRNA gene for the total bacterial community and six AmpC β -lactamase gene groups as representatives of ARG. The efficiency of the real-time qPCR reaction was 92% for the 16S rRNA gene with an R-squared value of 0.99. The percentage efficiencies for AmpC β -lactamase gene groups qPCR reactions were 95% for ACC, DHA, and LAT/CMY/BIL gene groups. 101% for MOX/CMY, 102% for FOX, and 103% for ACT/MIR gene groups. The R-squared values for all the reactions were between 0.98 and 0.99 for all the real-time qPCR reactions (Figure 29). The slopes of the reactions were between -3.301 to -3.528 for all the real-time PCR reactions. Figure 29 shows some of the standard curves generated with which all the unknown samples were compared. Samples that amplified below the threshold values were excluded from the analysis.

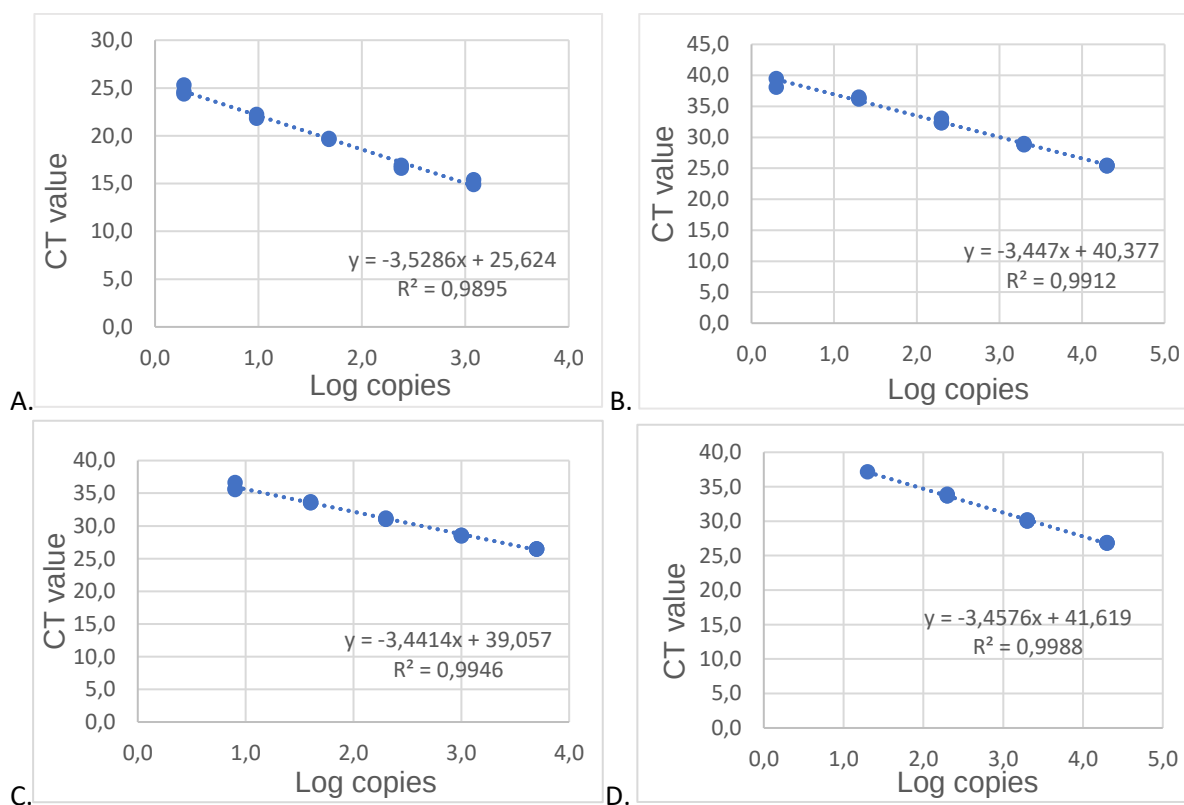


Figure 29: The depiction of the standard curves generated from real-time qPCR reactions. (A) 16S rRNA gene. AmpC β -lactamase groups (B) ACC, (C) LAT/CMY/BIL, (D) DHA.

4.9.1 The quantity of ARG/16S rRNA gene copies from microcosm samples

ARG specifically AmpC β -lactamase gene groups, were quantified to determine their quantity on the bacterial community found on substrates (microplastics and wood) and source water (river and WWTe). Microplastics isolated from the river microcosms showed a high average copy number of ARG, averaging 154.86 ± 109.50 gene copies/16S rRNA than in wood samples (91.96 ± 65.02 gene copies/16S rRNA) and water samples (2.79 ± 1.98 gene copies/16S rRNA). A similar pattern was observed in the substrates from the WWTe microcosms (Figure 30). The average total copy number of ARG in microplastic samples from the WWTe microcosm was 232.68 ± 164.53 gene copies/16S rRNA. This average was the highest quantity of ARG gene copies detected between the river and WWTe microcosms. The wood substrates contained 5.23 ± 3.70 gene copies/16S rRNA, followed by WWTe water samples with 0.17 ± 0.12 gene copies/16S rRNA (Figure 30).

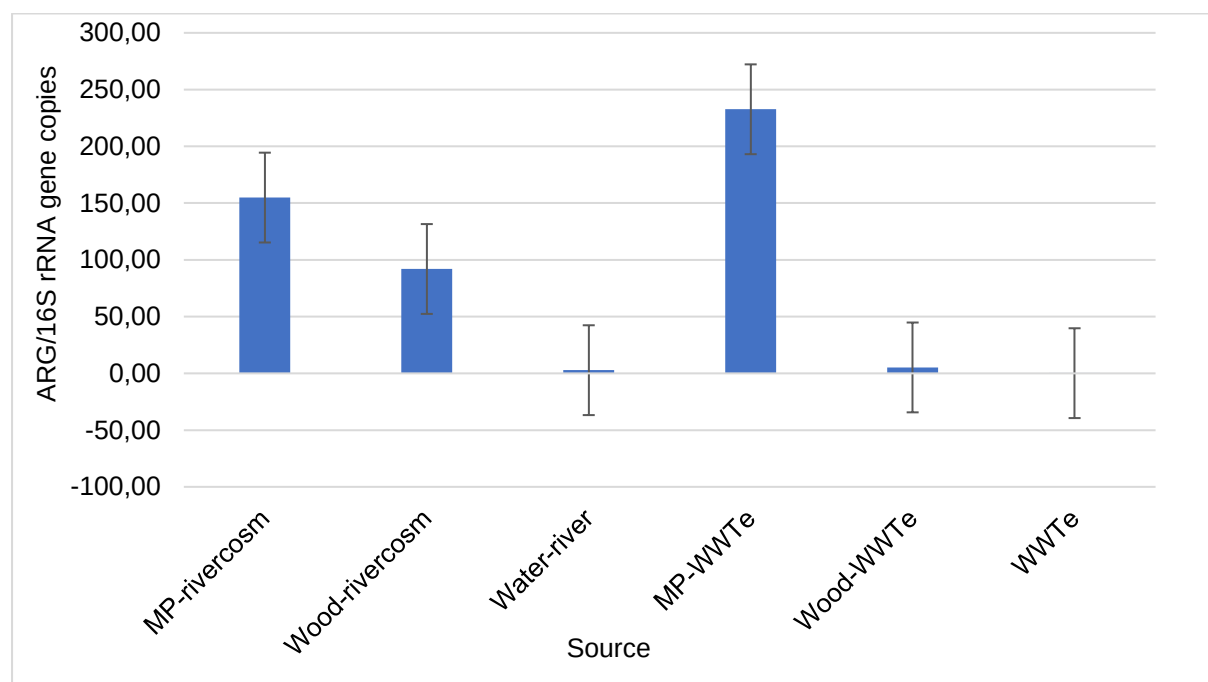


Figure 30: Graphical representation of the average total of ARG in gene copies/16S rRNA from microcosm experiments of both river water and WWTe.

4.9.1.1 Distribution of AmpC β -lactamase gene groups in microcosm samples

The AmpC gene groups ACC, MIR/ACT, and LAT/CMY/BIL were not detected during screening with end-point PCR. These gene groups were excluded for qPCR analysis. The AmpC gene group FOX was present in all samples from the river microcosm and the WWTe microcosm (Table 8). Furthermore, this group had the highest average for the total copy

number at 18.22 ± 12.18 gene copies/16S rRNA, followed by MOX/CMY (14.18 ± 10.09 gene copies/16S rRNA) and DHA (0.09 ± 0.06 gene copies/16S rRNA) from the microcosm experiments (Table 9). The highest copy number amongst the individual AmpC β -lactamases was observed in MOX/CMY gene group at 196.91 ± 139.24 gene copies/16S rRNA in the microplastic samples from the WWTe microcosm. This was followed by FOX with copy numbers of 99.33 ± 70.24 gene copies/16S rRNA from microplastic samples from the river microcosm. The AmpC gene group FOX showed a decrease in copy numbers in microplastics (99.33 ± 70.24 to 48.25 ± 34.12 gene copies/16S rRNA) and wood substrates (91.22 ± 64.85 to 0.06 ± 0.04 gene copies/16S rRNA) from the river microcosm starting from day 3 to day 14. A similar trend was observed in the WWTe microcosm with microplastic samples. The copy numbers detected in microplastic samples were 16.88 ± 11.94 gene copies/16S rRNA on day 3 which decreased to 0.26 ± 0.18 gene copies/16S rRNA after day 14 (Table 9). The copy number of the FOX gene in the surrounding water post-incubation was lower in WWTe (0.03 ± 0.02 gene copies/16S rRNA) and river water (2.79 ± 1.98 gene copies/16S rRNA). The DHA gene group was only found in wood fragments in WWTe at relatively low concentrations of 0.06 ± 0.04 gene copies/16S rRNA in samples isolated on day 7 and 0.20 ± 0.14 gene copies/16S rRNA on day 14 (Table 9).

Table 9: Average copy number of ARG (AmpC gene groups) quantified from microcosm samples (units in gene copies/16S rRNA $\pm$ standard deviation)

Sample	Substrate	Source	Period	ACC	DHA	FOX	LAT/CMY/BIL	MIR/ACT	MOX/CMY
F3	MP	River	D3	-	-	99.33 $\pm$ 70.24	-	-	-
F4wood	wood	River	D3	-	-	0.18 $\pm$ 0.13	-	-	-
WD3W7	MP	WWTe	D3	-	-	16.88 $\pm$ 11.94	-	-	196.91 $\pm$ 139.24
F3C	MP	River	D7	-	-	0.56 $\pm$ 0.40	-	-	-
F4wD	wood	River	D7	-	-	91.72 $\pm$ 64.85	-	-	-
WD7W9	MP	WWTe	D7	-	-	3.47 $\pm$ 2.46	-	-	11.10 $\pm$ 7.85
WD7W12	wood	WWTe	D7	-	0.06 $\pm$ 0.04	0.69 $\pm$ 0.49	-	-	3.75 $\pm$ 2.65
F14	MP	River	D14	-	-	48.25 $\pm$ 34.12	-	-	-
F15	MP	River	D14	-	-	6.72 $\pm$ 4.75	-	-	-
F16WD	wood	River	D14	-	-	0.06 $\pm$ 0.04	-	-	-
W17	MP	WWTe	D14	-	-	0.26 $\pm$ 0.18	-	-	0.28 $\pm$ 0.19
W18	MP	WWTe	D14	-	-	1.72 $\pm$ 1.22	-	-	2.05 $\pm$ 1.45
W19	wood	WWTe	D14	-	0.20 $\pm$ 0.14	0.53 $\pm$ 0.37	-	-	-
RW		River	D14	-	-	2.79 $\pm$ 1.98	-	-	-
WWTe		WWTe	D14	-	0.01 $\pm$ 0.01	0.03 $\pm$ 0.02	-	-	0.03 $\pm$ 0.02

(-) = undetected; MP (microplastic); WWTe (Wastewater treatment effluent)

4.9.2 The quantity of ARG/16s rRNA from environmental samples

In environmental samples (Figure 31), the average total of ARG copy number in microplastics (31.59 ± 6.63 gene copies/16S rRNA) was greater than in water samples from the river (7.83 ± 0.19 gene copies/16S rRNA). The WWTe water samples showed a high copy number of ARG with 612.77 ± 162.53 gene copies/16S rRNA detected in water samples than in microplastics (48.82 ± 4.40 gene copies/16S rRNA). The results indicated that the ARG copy number was high in WWTe microplastics than in microplastics from the river. Furthermore, the ARG copy numbers in WWTe were greater than in microplastics from the river and surrounding water combined.

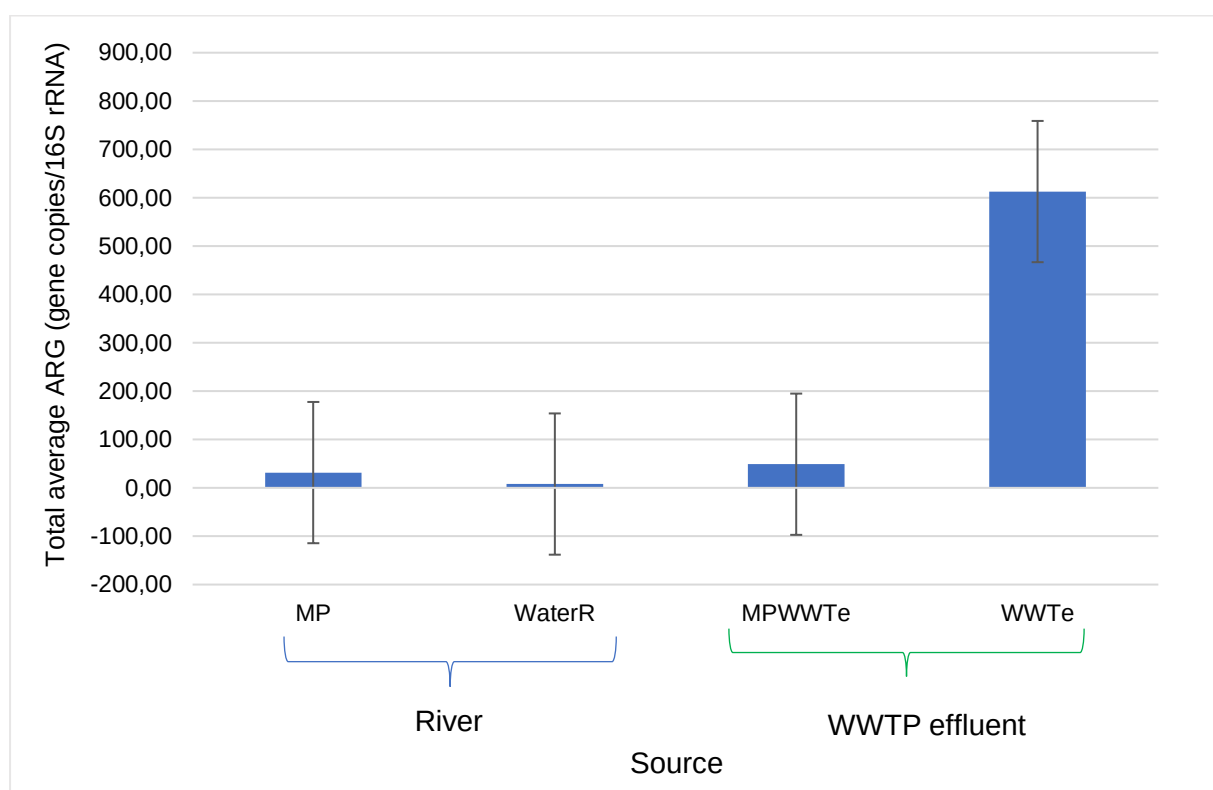


Figure 31: Graphical representation of the average total ARG (gene copies/16S rRNA) in microplastic (MP) and water samples from the Mooi River and WWTP effluent.

4.9.2.1 Quantification of AmpC gene groups from environmental samples

According to each respective site, the field samples were separated into two groups: microplastics and water (Figure 8 and Table 8). Six AmpC β -lactamase gene groups were quantified using real-time qPCR. The AmpC gene groups DHA, FOX, LAT/CMY/BIL, and MOX/CMY were not detected in samples from Site M1 and microplastic samples from Site O2 (Table 8). ACC and MIR/ACT gene groups were detected in all sites in both microplastics and water samples. Copy numbers of the ACC gene group ranged from 0.01 ± 0.01 gene copies/16S rRNA to 71.08 ± 50.26 gene copies/16S rRNA. MIR/ACT copy numbers ranged

from 0.06 ± 0.01 to 91.94 ± 103.90 gene copies/16S rRNA. From Table 10, the results indicated that the ACC gene groups' copy numbers in microplastic samples were greater than copy numbers detected in water samples from Site M1 ($0.28 \pm 0.20 > 0.01 \pm 0.01$ gene copies/16S rRNA), Site O2 ($0.17 \pm 0.12 > 0.12 \pm 0.12$ gene copies/16S rRNA), Site G3 ($0.75 \pm 0.53 > 0.16 \pm 0.12$ gene copies/16S rRNA), and Site T5 ($0.86 \pm 0.61 > 0.07 \pm 0.05$ gene copies/16S rRNA). In the MIR/ACT gene group, copy numbers in microplastic samples were greater than in water samples from Site M1 ($0.64 \pm 0.12 > 0.10 \pm 0.07$ gene copies/16S rRNA), Site O2 ($11.60 \pm 10.08 > 1.09 \pm 0.01$ gene copies/16S rRNA) and Site G3 ($1.97 \pm 4.07 > 0.06 \pm 0.01$ gene copies/16S rRNA). However, in Site W4 and Site T5 MIR/ACT gene copies in water samples were higher than in microplastic samples (Table 10).

Samples from Site W4 were collected from the WWTe that flows into the Mooi River. Water samples collected from the WWTe in Site W4 presented high copy numbers of AmpC β -lactamase gene groups than microplastics samples from all the sampling sites (Table 10). The highest copy numbers were observed in the LAT/CMY/BIL gene group with an average of 248.19 ± 304.99 gene copies/16S rRNA, followed by MOX/CMY (188.95 ± 370.57 gene copies/16S rRNA), MIR/ACT (91.94 ± 103.90 gene copies/16S rRNA), ACC (71.08 ± 50.26 gene copies/16S rRNA), FOX (10.97 ± 7.75 gene copies/16S rRNA), and DHA (1.65 ± 0.91 gene copies/16S rRNA) respectfully. In microplastic samples, collectively, the DHA gene group had the lowest copy number averaging at 0.12 ± 0.09 gene copies/16S rRNA. This gene group was also the least detected gene, as it was found in 2/5 sites (Table 10).

Table 10: Average copy number of ARG (AmpC gene groups) quantified from field environmental samples of microplastics and water (units in gene copies/16S rRNA $\pm$ standard deviation).

Sample	Site	Source	ACC	DHA	FOX	LAT/CMY/BIL	MIR/ACT	MOX/CMY
M1P	M1	MP	0.28 $\pm$ 0.20	-	-	-	0.64 $\pm$ 0.12	-
M1F		River	0.01 $\pm$ 0.01	-	-	-	0.10 $\pm$ 0.07	-
O2P	O2	MP	0.17 $\pm$ 0.12	-	-	-	11.60 $\pm$ 10.08	-
O2F		River	0.12 $\pm$ 0.12	1.20 $\pm$ 0.56	8.67 $\pm$ 2.38	0.46 $\pm$ 0.41	1.09 $\pm$ 0.01	0.77 $\pm$ 0.10
G3P	G3	MP	0.75 $\pm$ 0.53	-	11.92 $\pm$ 0.89	1.32 $\pm$ 0.24	1.97 $\pm$ 4.07	1.17 $\pm$ 0.04
G3F		River	0.16 $\pm$ 0.12	-	6.01 $\pm$ 1.32	0.02 $\pm$ 0.01	0.06 $\pm$ 0.01	0.09 $\pm$ 0.03
W4P	W4	MP-WWTe	15.94 $\pm$ 11.27	0.18 $\pm$ 0.13	3.33 $\pm$ 2.35	24.01 $\pm$ 5.42	1.01 $\pm$ 1.13	4.35 $\pm$ 4.78
W4F		WWTe	71.08 $\pm$ 50.26	1.65 $\pm$ 0.91	10.97 $\pm$ 7.75	248.19 $\pm$ 304.99	91.94 $\pm$ 103.90	188.95 $\pm$ 370.57
T5	T5	MP	0.86 $\pm$ 0.61	0.06 $\pm$ 0.05	-	-	0.24 $\pm$ 0.17	1.25 $\pm$ 0.45
TF		River	0.07 $\pm$ 0.05	0.01 $\pm$ 0.65	7.88 $\pm$ 0.04	0.17 $\pm$ 0.0	0.65 $\pm$ 0.58	3.12 $\pm$ 0.21

(-) = undetected; MP (microplastic); WWTe (Wastewater treatment effluent)

4.10 Summary of the results in chapter 4

The results in this study contained microcosm experiments and field environmental samples analysis for the characterization of the “plastisphere” (Zettler *et al.*, 2013) in a South African river system. The analysis of SEM images showed that when substrates are introduced into the medium (water), microorganisms will attach to the surface. The images showed microbial colonization after 48-hour incubation of microplastics and wood substrates in both river and WWTe microcosm vessels (Figure 10-16). The results further gave indicated that microbial colonization and the formulation of stable biofilms on microplastics is a slow process in low nutrient settings as opposed to nutrient-rich settings (WWTe). For example, in LD-PE samples in river water, microbial cells found on the surface were low on days 2 and 4, and only after day 14, an assembly of microbial cells was observed (Figure 11). In WWTe microcosm vessels, microbial cells were higher than in river water on day 2. An assembly of microbial cells was detected from as early as day 4. On day 7, a pre-mature developing biofilm was detected (Figure 14). The wood substrates showed high surface colonization by microbial cells from day 2. The overall observation is that the surface of the substrates may influence the level of colonization on the substrates. The more creviced or pitted the surface of the substrate was, the higher the microbial cell colonization.

The idea that the surface of the substrate plays a role can be seen on the images from the direct field sample collection (Figure 24). SEM images of samples collected directly from the river revealed a diverse community consisting of diatoms and microbial biofilms on the surfaces. The topographic view of the surface at 300 μm (Figure 24 group B) showed that the surfaces of the microplastics were creviced, and some spots were smooth. The spots that showed some pitting and roughage were covered with biofilms (Figure 24 group C). Field experiments also revealed that microplastics are abundant in the river and mostly dominated by fibres and solid fragments. Fragments, foams, and films were abundant in areas near recreational, industrial, and economic activities are high which is indicative of direct pollution from external sources. The presence of microplastics in WWTe indicates that microplastics are not removed from the WWTP during treatment, and these plastics are released into the environment.

The bacterial community composition (BCC) showed a dominance of common primary and secondary colonizers of substrates that is phyla Proteobacteria and Bacteroidota. From the microcosm samples, it was observed that the BCC changed over time. The alpha-diversity showed that the free-living microbial community in water was richer and diverse than the community composition in microplastics and wood substrates. From the environmental microplastics and water samples, the scenario was the same as observed in the microcosms,

the BCC on microplastics was less rich and diverse than in water. The core microbial abundance analysis showed that some genera (*Aeromonas*, *Legionella*, and *Pedobacter*) were more enriched on microplastics than the surrounding water community. Furthermore, some families were significantly higher (p-value < 0.05) on microplastics than in water samples and vice versa, this included the *Enterobacteriaceae* and *Legionellaceae* families (Tables 3-6). Some taxa were dominant in water samples than microplastics, such as the *Exiguobacterium* genus from the *Exiguobacteraceae* family, and the *Alteromonadaceae* family. Polymer type's community composition was only tested and applied in microcosm experiments since the microplastic polymer types were also known. The results showed that the BCC on PP substrates was richer and diverse but less dominant than in LD-PE and PS substrates.

Finally, the study determined that genes associated with antibiotic resistance could be detected on microplastics and river water samples. It was observed that the FOX, MOX/CMY, and DHA AmpC β -lactamase gene groups were present in the river water and WWTe from the 2019 and 2020 water sampling. From the field environmental samples, it was observed that ACC and MIR/ACT gene groups were present across the river system. The remaining AmpC gene groups (DHA, FOX, LAT/CMY/BIL, MOX/CMY) together with the *int1* gene were detected in the more urban impacted sites (Figure 8) in the river system. Real-time qPCR analysis showed that gene copy number was high in microplastics than wood and water from the river and WWTe microcosm. In the field samples, microplastics from the river showed high gene copy numbers than in water samples. However, the water samples from the WWTe (Site W4) showed high copy numbers than in the microplastics samples. The highest copy numbers were observed for LAT/CMY/BIL, followed by MOX/CMY, MIR/ACT, ACC, FOX, and DHA. DHA gene copy numbers were relatively low in both microcosm and field environment samples. Overall, the results indicated that microplastics harbour microorganisms (selectively) that carry resistance genes in the environment.

CHAPTER 5: DISCUSSION

As an integral part of a strategy to manage the potential threat posed by microplastics in the aquatic environment, it was imperative to provide a science-based risk assessment on the impact of microplastics in inland freshwater systems. Furthermore, the investigation of microplastics as a vector for support and transport of microorganisms and ARG is of necessity in freshwater systems. This research study aimed to shed light on microplastic-microorganism interactions in a river system in South Africa.

5.1 Microcosm experiments: cell colonization on microplastics and wood substrates

Microorganisms are known to attach themselves to and exist on particle surfaces in aquatic systems (Cunliffe *et al.*, 2011). Through survival mechanisms such as biofilm formation, they can withstand environmental stressors (Wiedenbeck and Cohan, 2011; Liao *et al.*, 2015). This study investigated the behaviour of microorganisms in river water and WWTe mediums, when sterile microplastics and wood substrates are introduced. SEM images viewed before incubation (Figure 9) and post-incubation (Figures 10-16) revealed that microbial colonization on substrates can occur within 48 hours. Furthermore, it was observed that microbial cells generally attached themselves near or around pits, grooves, and jagged edges of both microplastics and wood surfaces. This habit is consistent with research studies which indicated that the surface of the particles plays a role in supporting and promoting bacterial colonization (Liao *et al.*, 2015; Rummel *et al.*, 2017; Hossain *et al.*, 2019), during the early stages of biofilm development (Oberbeckmann *et al.*, 2018). While it is acknowledged that the substrates' surface increases the chances of bacterial attachment, this study also highlighted that the microbial cells' physical structures may also play a role in the attachment process. The role of bacterial physical and/or morphological structures' influence in attachment processes on environmental substrates has been debated amongst researchers (Liao *et al.*, 2015). However, the SEM images in this study showed that bacterial cell extensions, most likely to be fimbriae, may have played a role in the attachment of cells on the microplastics particles and wood substrates, especially around the pitted parts of the substrates.

In terms of different mediums (source water), substrates incubated in river water (Figures 11-12) were less colonized by microbial cells when compared to substrates in WWTe (Figures 13-16). This is not surprising considering that WWTe has a more nutrient load than river water (Raju *et al.*, 2018). Therefore, the rate and frequency of attachment to substrates in the medium would be elevated (Liao *et al.*, 2015). Additionally, bacterial community profiling indices showed that bacterial composition in WWTe was richer and more diverse than in river

water (Figure 19). The formation of aggregates was observed in the microcosm vessels that contained microplastic and wood substrates as compared to the control microcosm vessels with no microplastics and wood substrates (Figure 7). This observation may explain the rapid development of biofilms that were observed in microplastic and wood substrates as early as day 4 and day 7 in WWTe settings (Figures 13-16). In environmental settings, the concept of aggregation formation may explain why aquatic organisms ingest microplastics (Naidoo *et al.*, 2016). Nutrient build-up on the substrates may increase the substrates' nutritional content and can be mistaken for food by aquatic organisms (Rochman *et al.*, 2013; Steer *et al.*, 2017). Health defects ranging from gills and gut blockage, reproduction, inflammation, and oxidative stress (Avio *et al.*, 2015; Grigorakis *et al.*, 2017; Jin *et al.*, 2018) due to ingestion of microplastics have been reported in research studies. What remains understudied are the effects of microorganisms associated with microplastics on aquatic organisms after ingestion.

Oberbeckmann *et al.* (2018) reported that with high nutrient availability, there is the rapid formation of a conditioning film which leads to an accelerated primary and secondary biofilm development that is less substrate-specific. SEM images in Figure 13 from days 4, 7, and 14 provide support to the concept of Oberbeckmann *et al.* (2018). The surface of the PP polymers was covered by microbial cells which were covered by a thick layer that may be a conditioning film. This coating potentially induced the clustering and adherence of microbial cells on the surface, which resulted in rapid premature biofilm formation as observed on day 14. The wood substrates in this study were used as representatives of naturally occurring particles. Wood substrates were more colonized by microbial cells than microplastics substrates in both river water (Figure 12) and WWTe (Figure 15) microcosms. Moreover, alpha diversity indices showed that the BCC on wood substrates was more rich, diverse, and even than on microplastics (Figure 19). Wood structures have more folded layers and/or compartments than microplastics substrates, which is more than enough surface area for microbial organisms to attach and build up biofilms. The microplastics used in this study had smooth solid surfaces, except for PS substrates which were foams. LD-PE also showed high bacterial colonization with rapid biofilm development when compared to other microplastic types. Hossain *et al.* (2019) made similar observations about the greater bacterial abundance on LD-PE than HDPE and PS substrates in their study. It has been indicated that microplastic structural chemistry and hydrophobicity may play a role in enhancing bacterial attachment on microplastics. However, this concept was not within the scope of this study.

The concerning issue is that microplastics can last longer in the environment than naturally occurring particles such as wood (Zettler *et al.*, 2013). Although wood may present and support more bacterial communities than plastic particles on their surfaces, the durability, chemical content, and manufacturing properties of microplastics versus wood indicate that

microplastics are a high-risk factor (Horton *et al.*, 2017; Jiang *et al.*, 2018). The SEM results from the substrates used in the microcosm experiments (Figure 11-16), and the environmental microplastics samples (Figure 24) showed that microbial cells preferred to build up biofilms and attach at the areas where there was roughness, pitting, and crevices. Some shearing of the microplastics substrates was observed on some of the images where microbial cells conglomerated (Figures 11 and 14). Although this may have been coincidental, members of several bacterial and fungal groups have been shown to degrade polymer substrates (Russell *et al.*, 2011) and use microplastics as a source of carbon (Sudhakar *et al.*, 2008). Sekiguchi *et al.* (2011) and Zettler *et al.* (2013) made observations through SEM that suggested microplastic surface alteration by microorganisms (Oberbeckmann *et al.*, 2014). Researchers are yet to publish about the discovery and identification of the pathways involved in the breakdown of plastic polymers by microorganisms (Rogers *et al.*, 2020).

5.2 Bacterial community composition on microcosm samples

The change in the composition of microbial structure on substrates isolate from day 3 to day 14 indicated how microorganisms behave when different substrates are introduced. Early biofilm formation is highly dynamic, yet it is a similar process on all substrates in terms of attachment process and development (Dang and Lovell, 2009). The variability comes into play when certain groups of microorganisms dominate on the substrates as opposed to the surrounding water community, based on the ecological process and beneficial carbon source the substrate offers (Rinanda *et al.*, 2019). More concerning is that bacterial taxa associated with animal and human infections may be abundant on microplastics as opposed to the surrounding water community (Kelly *et al.*, 2021). The highlight from studies is that microplastics may selectively support the attachment of bacterial pathogens in different mediums (Wu *et al.*, 2019).

Members of the phyla Proteobacteria and Bacteroidota were constantly identified as the dominant groups in all samples from both the river water and WWTe microcosms. The top two phyla were reported as first and secondary colonizers of biofilms in substrates found in the marine system (De Tender *et al.*, 2015). A study by Oberbeckmann *et al.* (2014) reported phyla Bacteroidota, Proteobacteria, and Cyanobacteria as the most common microbial groups on microplastics from their results. These are phyla with a wide range of microbial members found in freshwater settings and include associated members of human and animal gut microbiota (Thomas *et al.*, 2011). The dominance of these two phyla observed on the early days of colonization (Figure 18) supports the notion reported in a study by De Tender *et al.* (2015), since microbial attachment on substrates may occur within a few hours of contact (Parrish and Fahrenfeld, 2019). The phyla distribution data (Figure 18) corroborates the

observations from SEM images (Figures 11-16), in which microbial community on early days was lower. The build-up of cells occurred as the incubation period extended. During this process, the dominance of bacterial taxa may differ from day 1 of incubation and the last day of incubation. The results revealed that a reshaping of the bacterial population structure occurred during the incubation period, which led to the introduction of other bacterial phyla such as Actinobacteria, Acidobacteriota, Patescibacteria, Firmicutes, and Cyanobacteria (Figure 18). The BCC on microplastics indicated that diverse bacterial communities were much more distinct across time than they were amongst each other (Lavery *et al.*, 2020). When comparing the microbial community on microplastics and wood, many taxa probably attach to microplastic opportunistically as a niche during the early stages of development and attach non-selectively to wood substrates. Community profiling results based on genus level of sample types showed that microplastics were less rich, diverse, and less even than water and wood substrates (Figure 19: A-C). McCormick *et al.* (2014) showed that microplastic biofilms were significantly less diverse than the microbial consortia in the surrounding water in an urban river.

The size difference between the microbial communities in water versus on microplastics is difficult to account for and should be considered when comparing the two groups (Debeljak *et al.*, 2017). This was evident from the DNA concentrations after extractions. The average DNA concentrations, quality, and quantity were lower on microplastics than in water samples (Figure 17). Wang *et al.* (2021) found evidence of the selection of microbial consortia on microplastics in the riverine system. In essence, the total BCC in water should be expected to be richer and more diverse than the microbial community on microplastics and wood substrates. In this study, what was observed is that certain bacterial groups were dominant on microplastics and wood substrates post-incubation during the microcosm experiments (Tables S1-A and S1-B). This indicated that colonization on microplastics may be selective, and microorganisms that can adapt and survive on microplastics surfaces prevail during biofilm conditioning (Miao *et al.*, 2019). The high abundance of OTUs on wood substrates compared to the different polymers (Figure 20) may be influenced by the surface and natural structural design of wood material (Guo and Wang, 2019). The visible folds and compartments on the wood substrates make it difficult to conclude whether the observed community was influenced by the composition of the wood substrate or it was due to some cells being trapped within the compartments/folds. This aspect could be explored.

5.2.1 Bacterial community membership dominance based on substrates and polymer type

Community membership was determined by performing biomarker analysis using LEfSe (Segata *et al.*, 2011) to observe the abundance of different genera on sample types (microplastics, water, and wood substrates) and substrate type from both river water and WWTe microcosms. The results from the microcosms showed higher enrichment of OTU sequences from genera *Aeromonas*, *Acinetobacter*, and *Roseomonas* on microplastics and wood samples than in the surrounding water (Table 3). The results raise alarming concerns as they reveal the ability of microplastics and wood substrates to be a niche for genera known to contain potentially pathogenic species, which can be transported across different areas in the river channels in environmental settings. Furthermore, members of these genera have been reported to cause serious health concerns such as bacteremia, lung, and gut infections in immuno-compromised patients in clinical settings (Rit and Sasha, 2012). Some species belonging to the above genera have genetic material that allows them to be resistant to a wide range of antibiotics (Kim *et al.*, 2016; Wong *et al.*, 2017; Ayoub Moubareck and Hammoudi Halat, 2020). As observed from Figure 21, some microbial groups at family level were dominant in the water medium than on the substrates and vice versa. This indicates that not all microorganisms thrive by attaching to the substrates.

The biomarker analysis showed that some bacterial taxa such as *Enterococcus*, *Enterobacter*, and *Nitrosomonas* (Tables 3-4 and Tables S1: A-B) were more enriched in water samples and wood substrates than on microplastics. Some bacterial taxa such as genera *Bosea* and *Roseomonas* were highly abundant on microplastics and wood samples from the river water microcosm. Conversely, in the WWTe microcosm, OTUs from genera *Bosea* and *Roseomonas* were not detected on microplastics but were detected in WWTe water samples (Table 3 and Table S1: A-B). This scenario may be a coincidental occurrence, and many free-living bacteria do not form biofilms. Thus, they do not steadily attach to substrates unless they are trapped by biofilm-forming organisms or trapped within the compartments of the substrates (Lavery *et al.*, 2020). Also, taking note of the surface structure of wood substrates versus microplastics substrates may play an important role in the colonization process (Rummel *et al.*, 2017; Oberbeckmann *et al.*, 2018). Some bacterial taxa that were enriched on the substrates are known to play a key role in microbial processes such as primary production and nutrient cycling (Falkowski *et al.*, 2008). For example, the genus *Zoogloea* was more enriched on microplastics and wood than in the surrounding water samples in both river water and WWTe microcosms. This genus is of aerobic chemoorganotrophic bacteria and plays an important role in the degradation of organic carbon and promotes floc formation in water

(McCormick *et al.*, 2014). This group may have contributed to the formation of floc aggregates observed in Figure 7. Additionally, OUT sequences belonging to the *Hyphomonadaceae* family (genus *Hirshicia*) were detected on microplastics and wood substrates (Table S1: A-B). The *Hyphomonadaceae* family contains members that have prosthecae and can produce polysaccharides holdfasts which enable them to attach firmly on to substrates. These structures can also partake in nutrient uptake for microorganisms from the surroundings (Abraham and Rohde, 2014; Oberbeckmann *et al.*, 2018). Microbial extensions and pili-like structures were visible and steadily attached to the surface of the microplastics and wood substrates (Figure 10-16).

5.2.2 Distribution of genera capable of biodegrading plastic

The greater scope of microplastic-microorganism interactions research studies is not only limited to the polymers as vectors of opportunistic pathogens but also to identify the activities within the plastisphere which may assist in mitigation strategies for the remediation and removal of microplastics in the aquatic environment (Zettler *et al.*, 2013). Although this was not the objective of this study but falls within the wide scope of microplastic research, it is imperative to highlight some of these features. Certain species such as those belonging to the genera *Pseudomonas* and *Ideonella* are reported to aid in the biodegradation of microplastics such as PE and PP (Shimao, 2001; Kathiresan, 2003) and utilize carbon as a source of energy. LEfSe results (Table S1-D) showed that genus *Pseudomonas* was present on all polymer types, and was higher in PS, followed by PP and LD-PE. This observation is consistent with the findings of McCormick *et al.* (2014) in which they suggested that this genus may be a common colonizer of plastic substrates. Furthermore, biomarker analysis showed that the species *Pseudomonas alcaligenes* was prevalent on microplastics. This species is known for the biodegradation of polycyclic aromatic hydrocarbons. It has also been reported to be an opportunistic pathogen (Suzuki *et al.*, 2013).

The results also showed that genus *Ideonella* was more abundant in microplastics than wood substrates from the WWTe microcosm. It has been demonstrated that *Ideonella sakaiensis* (a species of genus *Ideonella*) can degrade Poly (ethylene terephthalate) (PET), which is a common material for synthetic fibres, plastic bottles, and single-use food containers (Yoshida *et al.*, 2016; Cesa *et al.*, 2017; Seo *et al.*, 2019). In this study, genus *Ideonella* was found enriched on PS substrates, and no bacterial taxon numbers were found on LD-PE and PP substrates (Table S1-D). A member of the genus *Acinetobacter* (*Acinetobacter baumannii*) has also been reported to have the ability to degrade LD-PE (Pramila and Ramesh, 2015). Genus *Acinetobacter* was more abundant on microplastics and wood substrates than in water samples from both the river and WWTE microcosms (Table 3). In contrast to the findings of

Pramila and Ramesha (2015) in terms of the LD-PE polymer, the results showed that the genus *Acinetobacter* was highly enriched on PS, followed by PP and LD-PE (Table S1-D). This contrast can be explained by the difference in LD-PE type used between the two experiments. In this study's microcosm experiments, sterile solid spherical LD-PE polymers were incubated in water samples from the river source and WWTe. Conversely, in Pramila and Ramesha's (2015) experiment, film and powdered LD-PE polymers were incubated with the bacterial species. However, their results showed a substantial degradation of microplastics by this species. Some researchers argue that there are no specific microplastic-associated bacterial communities, but rather, some bacterial groups are influenced by the characteristics of the individual plastic polymer content, such as additives and content, which may lead to the deterioration of the plastic material (Pinto *et al.*, 2019). What is evident from the research studies regarding the ability of microorganisms to degrade plastic material is that this action is conducted by enzymatic contents of the organisms (Amobonye *et al.*, 2020). Many other bacterial and fungal organisms that can completely degrade different polymers in aquatic systems are yet to be discovered.

5.3 Field microplastic Mooi River system

The prevalence and persistence of microplastics in the aquatic environment present a cause for concern, because microplastics as vectors may potentially proliferate and spread opportunistic pathogenic organisms from freshwater to marine settings (McCormick *et al.*, 2014). Research studies on microplastics in the aquatic environment mostly focuses on the quantification of microplastics in marine environments (Zettler *et al.*, 2013). Studies on the identification and characterization of biofilm communities on microplastics, especially in river systems of South Africa are lacking. An objective of this study was to find microplastics in a river system and characterize the BCC on microplastics versus the bacterial community in surrounding water at each respective site. Moreover, the goal of the study was to compare the microcosm experiment results to the results of the microplastics directly isolated from the river system.

5.3.1 Microplastic pollution in the river system

Although quantifying microplastics was not our focus for this study, 206 microplastic particles were found in 1800 litres of filtered surface water across five sampling points. The microplastics samples were dominated by thread-like filamentous substrates known as fibres (77.2%), followed by fragments (9.7%), foams (7.3%), films, and microbeads (2.9%). Research studies that have sampled microplastics from freshwater systems found that fibres and fragments were the most detected microplastic groups found in freshwater settings (McCormick *et al.*, 2014; Bouwman *et al.*, 2018; Luo *et al.*, 2019; Wang *et al.*, 2019). Most of

the fibres found across all five sampling points were synthetic and may have originated from clothing material. The investigations by Browne *et al.* (2011) and De Falco *et al.* (2019) revealed that a clothing garment can produce more than 1500 fibres per wash (Cesa *et al.*, 2017). This finding may shed light on the reason behind the prevalence of fibres across river systems, considering that microplastics can be transported from a one-point source to other parts of the river.

In addition to the above mentioned, fragments, films, and foams are primarily from the degradation or breakdown of larger parts of plastics such as plastic water bottles, food packaging containers, and wrappers. In this study, these forms of microplastic types were found in the urban parts of the river from Sites O2, G3, W4, and T5 (Figure 8). Specifically, at Site G3, fragments, foams, and films were dominant. This is a site that is exposed to high pollution impact from places such as restaurants and recreational facilities which utilize plastic materials for packaging food and clothing items for consumers. Most notably, the Mooi River flows through the mall and restaurants. Therefore, all the illegal dumped plastic material ends up passing or pilling from Site O2 to Site T5. This scenario presents a problematic issue as foodborne organisms such as *Bacillus spp.* and *Escherichia spp.* (Bintsis, 2017), may proliferate on microplastics and be transported further to downstream areas of the river and may have health implications on both humans and aquatic animals.

5.3.2 Microbial life on microplastic surface isolated from the river

Microplastics recovered from the Mooi River were aged and were densely colonized by microbial and planktonic cells. SEM images (Figure 24) provided evidence of possible active growth of cells and possibly some symbiotic relations, a presence of rich eukaryotic and bacterial microbiota on the solid substrates were observed on the substrates. Zettler *et al.* (2013) made similar observations from their microplastic samples and regarded the community as the “plastisphere”. The surfaces of the microplastics showed degradation, crevices, and pitting (Figures 22 and 24). These are the spots where biofilms were most dominant when viewing the aerial surface of the polymers (Figure 24: group B). These findings are consistent with what was observed from the results of the microcosm experiments and support the phenomenon of microbial cell attachment and biofilm development onto substrates in aquatic systems (Liao *et al.*, 2015). Furthermore, microbial cells of different shapes, including rods, filaments, and cocci were abundant on the surface of the microplastics. The microplastics from the river showed high patches of biofilms on the surfaces covered with a slime-like coating, which is to be expected since the biofilm communities protect themselves through this covering layer (Flemming *et al.*, 2016). 16S metabarcoding enabled the identification of the bacterial groups that were dominant of the microplastics substrates such

as the genera *Bacillus*, *Citrobacter*, and *Streptococcus* (Tables S1: A-B). These are all known to show the morphological structures identified on the microplastic surfaces using SEM (Figure 24) (Madigan *et al.*, 2014).

5.3.3 Characterization of the microbial community in field

microplastics and water

It has been reported that the BCC varies across specific points of the river system due to various impact factors such as geographical location and anthropogenic impact at each respective site (Jordaan and Bezuidenhout, 2016). In this study, microplastics were isolated from the river to observe and compare the BCC on microplastics and free-living communities in the water at each respective sampling point. Overall, the samples were dominated by phylum Proteobacteria as expected, as it is one of the major phyla with a wide range of pathogenic and non-pathogenic members (Rizzati *et al.*, 2017). This was followed by Bacteroidota as the second-largest dominant phyla. These two major phyla contain members known to be common inhabitants of microplastic substrates (De Tender *et al.*, 2015). Moreover, microplastic samples were dominated by these families: *Enterobacteriaceae*, *Diplorickettsiaceae*, *Legionellaceae*, and *Acetobacteraceae*. The overall water samples were dominated by *Alteromonadaceae*, *Moraxellaceae*, *Exiguobacteraceae*, and *Pseudomonadaceae*. It can be deduced that microplastics may shape the bacterial groups leading to a different bacterial profile between microplastics and the surrounding water (McCormick *et al.*, 2014).

The results in this study also showed that the BCC varied across the sites and microplastics samples from each sampling point. For example, in Site M1, with the exclusion of Proteobacteria and Bacteroidota phyla, the third top phylum was Actinobacteriota (14%) in microplastic samples and Firmicutes (8%) in water samples. Whereas in Site G3, the second dominant phylum in microplastics was Patescibacteria, followed by Bacteroidota. In the water samples, the second dominant phylum was Bacteroidota, followed by Planctomycetota (Figure 25). This difference in BCC can be attributed to many factors such as geographical location, environmental conditions such as pollutants, microbial processes, and polymer type (Oberbeckmann *et al.*, 2018; Jordaan *et al.*, 2019). Bacterial phyla in Site W4 and T5 were similar but differed in terms of dominance. Further analysis showed that wastewater-related bacterial taxa were present on microplastics from Site W4 and Site T5. It is to be noted that when microplastics enter the river systems from various sources in environmental settings, they may be inhabited already by microbial groups and other organic and inorganic matter. Microplastic biofilms in WWTP are not destroyed but are modified, which leads to the

introduction of wastewater-related microbial groups on the already formed bacterial groupings on the microplastic surfaces (Kelly *et al.*, 2021).

5.3.4 Microbial community profiling of the field environmental microplastics

Microbial diversity on microplastics reportedly differs from the surrounding water and has been observed in marine water-related studies (Zettler *et al.*, 2013; Bryant *et al.*, 2016) and freshwater studies (McCormick *et al.*, 2014). This study made similar observations in the microcosm studies and environmental samples. The microcosm experiments revealed that bacterial diversity in the surrounding water was richer, diverse, and more even than the microbial community on microplastics (Figure 19). The bacterial diversity from the field samples showed that the microbial community in water samples was more rich, diverse but less even than on microplastics (Figure 26). However, the difference in diversity was not significant as the p-value for the diversity indices was greater (p-value > 0.910) than the statistical set value $p < 0.050$. The challenge with comparing the microplastic community versus the surrounding water can be attributed to the size difference of the samples and bacterial dominance, considering that microplastics are relatively small (< 5 mm) compared to the filtered water. The biofilm community on the surfaces would be dominated by specific groups of taxa that perform one function other than the heterogeneous bacterial groups in the surrounding water. Moreover, because the river is constantly flowing and receives direct and indirect anthropogenic pollutants from the surrounding land-based sources, it means that as microplastics are transported from one area to the next, more microbial agents are introduced into the biofilm community on the substrates (De Tender *et al.*, 2015; Kelly *et al.*, 2021).

5.3.5 Community membership distribution on microplastics versus water from the river

A recent study by Mughini-Gras *et al.* (2021) reiterated the notion that microbial community on microplastics is unique and that taxon selection occurs during biofilm development. Some earlier studies conducted have shown that there are some taxa-specific groups solely found on microplastics from the surrounding water in microcosm studies and field studies (Zettler *et al.*, 2013; McCormick *et al.*, 2014; Miao *et al.*, 2019). Most interesting is that some specific microbial assemblages form on different polymers within the water column, i.e., laboratory incubation or *in situ* incubation experiments (Kesy *et al.*, 2019; Pinto *et al.*, 2019). This indicates that there is some selective enrichment of microbial assemblages taking place on the microplastic surfaces in the water column (Wang *et al.*, 2020). This concept was also observed from the microcosm experiments, where certain members were more dominant on

microplastics and wood substrates than in water samples from both the river water and WWTe microcosms.

The core community membership distribution and bacterial taxa dominance between microplastics and water were analyzed from family level (Figure 27 and Table 5) and genus level (Table 6) with LEfSe biomarker analysis (Segata *et al.*, 2011). The results showed that some families such as *Bacteroidaceae*, *Enterobacteriaceae*, and *Legionellaceae* (Table 5), were significantly higher ($p < 0.05$) on microplastics than in water samples, except for the *Exiguobacteraceae* and *Alteromonadeceae* families which were significantly higher (p -value < 0.05) in water samples (p -values for individual taxa on Table 5). Oberbeckmann *et al.* (2018) showed that OTUs belonging to the *Enterobacteriaceae* family were more enriched on microplastic samples than in the surrounding water. The results from this study in both microcosm experiments and field samples (Tables 3-6) showed that genera such as *Citrobacter*, *Enterobacter*, and *Escherichia_Shigella* from the *Enterobacteriaceae* family were higher on microplastics than in the water. Moreover, these genera (*Citrobacter*, *Enterobacter*, and *Escherichia_Shigella*) were more enriched on microplastics samples found at Sites O2, G3, W4, and T5. It is reported that members belonging to the above-mentioned genera are commonly found in animal and humans' intestinal microbiota, which indicates faecal contamination in water (Jacoby, 2009). The site from where the samples were isolated, shows a greater occurrence of sewage contamination and animal excreta from the surrounding animal husbandry areas entering the river stream (Figure 8 and Table S2-B) (Jordaan *et al.*, 2016).

The results showed a high abundance of genera such as *Bacillus*, *Ideonella*, *Legionella*, *Pedobacter*, *Staphylococcus*, *Xanthobacter*, and *Zoogloea* on microplastic samples, from WWTe in Site W4 including the downstream Site T5 samples (Table S2-B). This finding highlights the two concerns which are of global concern in research studies. Firstly, microplastics are not removed or prevented from escaping WWTP (Kay *et al.*, 2018). Secondly, WWTP-related organisms and clinically relevant pathogenic organisms are released within biofilms on the surfaces of microplastics and are dispersed to downstream parts of the river (Lin *et al.*, 2018; Kelly *et al.*, 2021). What is also concerning is that the species belonging to the genera *Staphylococcus*, *Enterobacter*, and *Pseudomonas* (which were enriched on microplastics) are part of the group known as ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter spp.*). These are on the WHO's priority list (Benkő *et al.*, 2020), as they are routinely reported as causes of life-threatening infections in clinical settings. Moreover, these members can protect themselves against the biocidal effects of antibiotics and are able to acquire and disseminate ARG in highly favourable environmental

conditions such as highly polluted river water and WWTP, and most frequent potentially within microplastic biofilms (Molale and Bezuidenhout, 2016; WHO, 2017; Mulani *et al.*, 2019; Wang *et al.*, 2020).

McCormick *et al.*'s (2014) research study was among the first to explore microbial communities on microplastics in a river system. They reported that there was significant colonization by wastewater-related taxa, such as the *Campylobacteraceae* family and the genus *Zoogloea* on microplastics isolated from the WWTP and microplastics may be a novel vector transporting pathogenic bacteria into the receiving water body. In contrast to McCormick *et al.*'s (2014) study, the results from this study from both the microcosms (Table 3) and field samples (Table S2: A) showed that OTUs from genus *Zoogloea* were more enriched on microplastics than in river and WWTe. Caution is expressed with regards to reporting on the pathogenic members in water from this study because the target fragment analyzed was relatively short. However, the high abundance of bacterial taxa known to have pathogenic members (*Bacteroides*, *Citrobacter*, *Kosakonia*, and *Arcobacter*) on microplastics, most notably in WWTe (Table S2-B), indicated that microplastics are possible reservoirs and vectors for opportunistic pathogens, wastewater-related organisms, including antibiotic resistance between resistant and non-resistant microorganisms. This emanated from the study's results as it was observed that most of the identified genera such as *Pedobacter* (Viana *et al.*, 2018) and *Rheinheimera* (Chen *et al.*, 2010) enriched on microplastics have been reported to carry multiple resistance genes even though they are not pathogenic (Vaz-Moreira *et al.*, 2014). Moreover, river streams and WWTP contribute to the dissemination of these microplastic-associated communities to the downstream parts of the river (Kay *et al.*, 2018).

In contrast to this study's microcosm results (Table 3 and 4), OTUs from the field microplastic samples showed that genera *Aeromonas* and *Acinetobacter* were more abundant in the surrounding water than in microplastic samples (Table S2-A). Many factors influence the colonization of microplastics by microorganisms, and the dynamics are much more complex in the environment. Microcosm studies aim to mimic what may potentially occur in environmental settings. This means that certain factors are controlled, and this brings about limitations and ambiguity of the studies. However, it serves as a benchmark for understanding certain patterns which occur in the environment. This study identified that nutrient load and water recycling in microcosm vessels may have been a limitation in understanding whether attachment on substrates by certain groups changes with different nutrient loads. However, like the observations of Pinto *et al.* (2019) and Lavery *et al.* (2020), some bacterial taxa may have attached opportunistically on microplastic substrates. It is challenging to understand the fundamentals of colonization by microbial groups in the environment due to the ecosystem's complexity. Studying certain individual species may provide an insight into understanding

colonization on substrates, but this would require many disciplines and a broader scope of the study.

5.4 Antibiotic resistance genes and microplastics

Antibiotic resistance continues to remain a major global health threat (WHO, 2017). In the future, antibiotic therapy may become ineffective unless mitigation strategies aim to address agricultural, environmental, and clinical factors contributing to the intensification of antibiotic resistance are addressed (Coertze and Bezuidenhout, 2020). Aquatic environments act as reservoirs of ARG and can simultaneously disseminate ARG and ARB across the water column (Marti *et al.*, 2014; Holmes *et al.*, 2016). Subsequently, microplastics have emerged as the current persistent pollutant. They could potentially be a hotspot for HGT and a vector of microorganisms and ARG, which can be disseminated to other downstream parts of the river, including the ocean (Arias-Andres *et al.*, 2018a). The extent to which microplastics impact South African river systems that harbour biofilms carrying ARG and ARB is unknown (Bouwman *et al.*, 2018). The last objective of this study was to explore the prevalence of ARG (specifically six AmpC β -lactamase gene groups) and the class 1 integrase gene (*intl1*) on microplastics. Furthermore, the detected AmpC β -lactamase gene groups were quantified to determine the number of gene copies in the total bacterial community from both microcosm and environmental samples.

5.4.1 Detection and quantification of genes from microcosm

experiments

Thus far, through microcosm experiments, this study has demonstrated that introducing sterile microplastics and wood substrates into the water column with free-living microorganisms results in the colonization of these newly introduced substrates. The study further observed that biofilms extensively covered microplastics in the river system and had bacterial and eukaryotic organisms present on the surface (Figure 24), suggesting some possible symbiosis on the microplastic surface (Zettler *et al.*, 2013). Water is a reservoir for microorganisms and facilitates the proliferation of genetic material with the help of mobile genetic elements (Bergeron *et al.*, 2015; Antelo *et al.*, 2018). In biofilm communities, this transfer of genetic information that includes resistance genes may be highly proliferated (Marti *et al.*, 2014; Arias-Andres *et al.*, 2018a). In the results from the microcosm experiments conducted in 2019, it was observed that only the FOX gene group was present in microplastics, wood, and filtrate water samples from the river water microcosm. In the WWTe microcosm, 3/6 ARG (FOX, MOX/CMY, and DHA) were detected from the AmpC β -lactamase gene groups. Two AmpC gene groups (FOX and MOX/CMY) were present in microplastics, wood, and filtrate water

samples, while DHA was only found in wood and filtrate water samples (Table 7). Aquatic environments are known to contain a variety of pathogenic and non-pathogenic microorganisms which carry ARG. The presence of ARG in water is to be expected as river systems are known to be reservoirs of ARG and ARB (Baquero *et al.*, 2008; Marti *et al.*, 2014). Furthermore, the *intl1* gene was only detected in microplastics, wood, and water samples from the WWTe microcosm. The *intl1* gene is generally found in environmental settings that are impacted by anthropogenic pollution. This gene has been used as a marker of pollution in aquatic settings (Gillings *et al.*, 2014). The presence of the *intl1* in aquatic settings is usually in association with a variety of ARG. WWTe is highly concentrated with microbial load and antibiotic residues and is generally richer in nutrients than river water (Pantanella *et al.*, 2020). This may explain the difference in the number of genes detected in the river water (1/7) versus WWTe (4/7) as shown in Table 7.

Microorganisms survive on substrates by the formulation of biofilms, made up of diverse and metabolically complex microbial consortia (Høiby *et al.*, 2010). It is reported that biofilms may contribute to antibiotic resistance, and this may be due to: (a) high cell density, (b) the proximity of cells, and (c) accumulation of mobile genetic elements such as plasmids with gene cassettes i.e., the *intl1* gene within the biofilm and the free-living microbial community (Marti *et al.*, 2014; Li *et al.*, 2019; Wu *et al.*, 2019). Yang *et al.* (2019) reported higher ARG levels on microplastic biofilms than in the surrounding water microbial community in marine settings. This study observed the same trend during the microcosm experiments (Figure 30). High copy numbers were found on microplastics samples from both river water (154.86 ± 109.50 gene copies/16S rRNA) and WWTe (232.68 ± 164.53 gene copies/16S rRNA). This was followed by wood substrates with an average copy number of 91.96 ± 65.02 gene copies/16S rRNA from the river water microcosm samples and 5.23 ± 3.70 gene copies/16S rRNA from the WWTe microcosm samples. The ARG gene copies in the surrounding water contained an average of 2.79 ± 1.98 gene copies/16S rRNA in river water and 0.17 ± 0.12 gene copies/16S rRNA, less than the substrates ARG copy numbers.

The microcosm experiments in this study support the notion that ARG levels on substrates may depend on microbial community composition and activity (Yang *et al.*, 2019). The results revealed that as the microbial composition was restructured due to biofilm development (Figure 18), there was a fluctuation in the ARG gene copy numbers (Table 9). For example, it was observed that FOX gene copies declined from day 3 to day 14 in both microplastics from 99.33 ± 70.24 to 48.25 ± 34.12 gene copies/16S rRNA and in wood substrates from 91.22 ± 64.85 to 0.06 ± 0.04 gene copies/16S rRNA. A similar trend occurred in microplastics and wood substrates incubated in the WWTe microcosm (Table 9) for FOX and MOX/CMY AmpC gene groups. The difference in gene copy numbers between the substrates and surrounding

water can be due to the selective nature suggested to occur in microplastic biofilms and the change in bacterial structure over time, or due to the cell death of ARG carriers, which may have occurred (Lavery *et al.*, 2020; Wang *et al.*, 2020). Arias-Andres *et al.* (2018a) reported that microplastics may influence the development of microbial communities, and this may influence the exchange of genes, including ARG, between different bacterial taxa. The design of the experiments was in a way that the water used was not cycled and no new fresh water with nutrients was added over the 14 days which limited the study. As a result, the nutrients may have been used up by microorganisms, allowing a certain group of microbes to dominate on the substrates.

5.4.2 The prevalence of genes from field microplastics and surrounding water

Following microcosm experiments, microplastics and water samples collected from the environment across five sites along the Mooi River in the NWP were screened for the presence of the six AmpC β -lactamase and the *int1* gene. ARG were quantified using real-time qPCR to determine the gene copies on microplastics versus the surrounding water at each respective site after screening. The results (Figure 31) showed that the average total gene copy number of ARG on microplastic samples from the river (31.59 ± 6.63 gene copies/16S rRNA) was greater than the average total gene copy number of ARG in water samples (7.83 ± 0.19 gene copies/16S rRNA) as it was observed in microcosm experiments (Figure 30). This can be explained by the proliferated exchange activity of genetic material between microorganisms in biofilms (Antelo *et al.*, 2018).

As already mentioned, biofilms on microplastics from the environment may already be heavily colonized by microbial cells from different sources (households, food, and packaging) when entering the river system. Also, the frequency of transfer in the “plastisphere” (Zettler *et al.*, 2013) would be relatively high and frequent particularly under favourable conditions where there is a constant feed of nutrients from the surrounding anthropogenic activities (Arias-Andres *et al.*, 2018b). Yang *et al.* (2019) reported on the incidence of microplastic biofilms containing high levels of ARG than water samples. Although there are some limitations with regards to qPCR in terms of determining the levels of ARG between microplastics and water samples, there is some indication that microplastics may be vectors of ARG and can transport ARB and ARG to various parts of the river (Arias-Andres *et al.*, 2018a; Eckert *et al.*, 2018; Wang *et al.*, 2021)

The samples isolated from the WWTe from Site W4 showed higher gene copy numbers in water (612.77 ± 162.53 gene copies/16S rRNA) than on microplastics samples (48.82 ± 4.40

gene copies/16S rRNA), which was opposite to what was observed in microcosm experiments (Figure 30). This result is to be expected, and it is also well recorded that WWTe contains higher levels of ARG and ARB due to the tremendous microbial and antibiotic residue content that exists in WWTP systems (Rodriguez-Mozaz *et al.*, 2015). Furthermore, the retention time at the WWTP could also explain the high gene copy numbers in WWTe than in microplastics (Vollertsen and Hansen, 2017; Coertze and Bezuidenhout, 2019). It is to be noted that the gene copy numbers of the ARG in WWTe microplastics were higher than that of microplastics and river water samples from the river (Figure 31). WWTP are reported as reservoirs and hotspots for increased gene transfer among microorganisms due to increased nutrient load and the influx of hospital effluents which contain common carriers of AmpC β -lactamase gene groups (Marti *et al.*, 2014) and other multi-drug resistance carrying bacteria. The mixture of all these different influents results in the transfer of genetic material between planktonic bacteria and biofilms on microplastics. The fact that this study identified biofilm growth on microplastics from the field samples (Figure 24 and S1-A), shows that the WWTP treatment processes do not destroy the microplastic biofilms. Therefore, the microplastics escape with wastewater-related microorganisms and a pool of resistance genes from the WWTP.

5.4.3 Prevalence of AmpC β -lactamase genes and the *int1* gene in the Mooi River system

On Microplastics and water samples from Site M1 2/6 ARG (ACC and MIR/ACT AmpC gene groups) were detected. 5/6 AmpC gene groups (ACC, DHA, MOX/CMY, MIR/ACT, LAT/CMY/BIL) in Site O2 and the *int1* gene were detected in water samples (Table 8). However, on microplastic samples from Site O2, only 2/6 ARG were detected as compared to the 6/6 ARG that were detected in the filtrate water samples. The two AmpC gene groups (ACC and MIR/ACT) that were detected in Site M1 were the same as those genes detected on microplastics from Site O2 (Table 8). Furthermore, the gene groups' copy numbers were higher on microplastic samples than in the surrounding water (Table 10). Reports have indicated that microplastics serve as vectors in aquatic environments. Considering that rivers are not actually stagnant but flow continuously, there is a great possibility of transportation of microplastics to downstream parts of the river (Laganà *et al.*, 2019). Based on the ARG profiling on microplastics found at Site M1 and Site O2 (Table 8), it is suspected that these microplastics may have been from a one-point source and/or plastic item. Moreover, Site M1 is more pristine than other sites as it is mainly impacted by agricultural and animal farming. In contrast, Sites O2, G3, W4, and T5 are affected by activities such as agricultural farming, animal husbandry, urban, industrial, and recreational activities (Figure 8). These are the sites that showed a diverse pool of AmpC β -lactamase and the *int1* gene presence.

It is proposed that BCC on microplastics, including the resistance gene profiles may be influenced by geographical factors (Oberbeckmann and Labrenz, 2020) including anthropogenic pollution (Karkman *et al.*, 2019). Coertze and Bezuidenhout (2020) highlighted that in densely populated areas in the Crocodile West River, there were high AmpC gene copy numbers, which may be due to influences from faecal pollution and antibiotic usage in those surrounding areas. An almost similar concept was observed in this study. The Sites O2, G3, W4, T5 were densely populated (more urban) and prone to anthropogenic pollution (Figure 8) showed a high detection rate of AmpC β -lactamase genes (Table 8). For example, 5/6 ARG (AmpC gene groups) were detected in Site G3 in both microplastics and water samples (Table 8). The ARG (AmpC gene groups) copy number was greater in microplastics than water samples, ranging from 0.75 ± 0.53 to 11.92 ± 0.89 gene copies/16S rRNA. In filtrate water samples gene copies ranged between 0.02 ± 0.01 to 6.01 ± 1.32 gene copies/16S rRNA. Site G3 is located where there are strong anthropogenic influences such as high urbanization, industrial activity, agricultural and recreational activities (Figure 8). Precedent to the above mentioned is that the water passes through the shopping centre, bird, and animal sanctuaries. Therefore, the prevalence of these AmpC β -lactamase gene groups in the river system could be related to faecal pollution impact from these sources (Coertze and Bezuidenhout, 2021).

In site W4 6/6 AmpC gene groups and the *int11* gene were present in both microplastics and water samples. This was expected because WWTP are not equipped well enough for the complete removal of ARG, microorganisms, and microplastics (Laht *et al.*, 2014; Raju *et al.*, 2018; Coertze and Bezuidenhout, 2020). Furthermore, biofilms on microplastic surfaces would make it difficult for the disinfection of all microorganisms passing through the treatment processes. Gene copy numbers were higher in the surrounding water samples ranging from 1.65 ± 0.91 to 248.19 ± 304.99 gene copies/16S rRNA, whilst in microplastics from Site W4 copy numbers ranged from 0.18 ± 0.13 to 24.01 ± 5.42 gene copies/16S rRNA. The great concern is that these samples were collected from the WWTe that had already been treated and was being released to the environment. It is understood that WWTe first goes to the maturation point that is near a bird sanctuary. The alarming concern is that birds that forage there would ingest microplastics harbouring pathogenic and non-pathogenic microorganisms and even those that contain ARG and may be deposited to new environments by faecal deposition from the birds into the environment. More concerning are the possible adverse health effects that would arise from microplastic ingestion by the animals (Guo and Wang, 2019; Harrison *et al.*, 2018). Microorganisms released from the WWTe may resuscitate when nutrients are available and above the threshold of the WWTP disinfectant. Moreover, biofilms on microplastic mostly escape untouched and can be transported to downstream parts of the river.

On Site T5 located downstream of Site W4, 5/6 ARG from the AmpC gene groups and the *int11* gene were detected in water samples, and only 4/6 ARG were detected on microplastics. The frequency of detection of ARG is still very high, as it confirms that WWTe contributes to the dissemination of ARG towards the downstream sites. It is however noted that microplastics are floating and bacterial assemblages may lose resistance genes over time in the environment as the biofilm community restructures (Kümmerer, 2009). It can also be noted that the impact of the flowing river (Loop Spruit) may increase the flow rate towards the site and also dilute the flowing water from upstream sites towards Site T5 (Figure 8), which would explain the relatively low gene copy number in Site T5. Sampling beyond Site T5 would have been beneficial for this observation because it stretches out of the more urbanized part of the city to more agricultural and rural settings.

The *int11* gene was prevalent from Sites O2, G3, W4, and T5 and samples O2F, G3P, G3F, W4P, W4F, and T5F where more than five AmpC gene groups were detected. The sites are prone to high pollution impact (Figure 8). The presence of the *int11* gene suggests that there is a greater chance of ARG being incorporated as part of the integrase-integron gene cassettes, heightening the possibility of gene transfer and overexpression as a result of selective pressure from pollutants (Marti *et al.*, 2014; Kaushik *et al.* 2018). However, it is noted that other mobile genetic elements may be contributing and facilitating the transfer and exchange of ARG between microplastic biofilms and free-floating microorganisms by HGT (Arias-Andres *et al.*, 2018a; Eckert *et al.*, 2018). Coertze and Bezuidenhout (2018) detected the AmpC and the *int11* genes on genomic and plasmid DNA. They reported that this may suggest a high risk of HGT and the potential dissemination of ARG to other parts of the aquatic system. This study detected the same resistance genes on the bacterial communities from microplastics and the surrounding water. The results indicate that microplastics may contribute to the process of HGT, which is proliferated in microplastic biofilms between microorganisms that carry these genes and those without these genes. Moreover, microplastics also contribute to the dispersal of ARG and ARB to other parts of the river stream. While microplastics travel through the water column, there is the continuous exchange of genes via mobile genetic elements (such as plasmids, transposons, and integrases) within biofilms and the surrounding water, which may result in increased copy numbers within the plastsphere, especially when there is semi-continuous input of resistance genes in the water body (Wang *et al.*, 2021).

AmpC β -lactamase gene groups (ACC, DHA, FOX, LAT/CMY/BIL, MIR/ACT, and MOX/CMY) are regarded as plasmid-mediated genes, as they were initially detected in bacterial plasmids (Aryal *et al.*, 2020). Over the years, they have been reported as the common group associated with antibiotic resistance in clinical isolates belonging to *Enterobacteriaceae* (Mohd Khari *et al.*, 2016; De Angelis *et al.*, 2020). Moreover, they are also associated with other mobile gene

elements particularly in environmental settings (Taylor *et al.*, 2011; Coertze and Bezuidenhout, 2019). In this study, it was observed that the *Enterobacteriaceae* family was prevalent in both microplastics and water samples. The highest copy number on AmpC gene groups were on LAT/CMY/BIL (45.70 ± 51.85 gene copies/16S rRNA), followed by MOX/CMY (28.53 ± 53.74 gene copies/16S rRNA) and MIR/ACT (10.93 ± 12.01 gene copies/16S rRNA). It is a cause for concern that the same AmpC β -lactamase groups that are prevalent in clinical settings are presenting themselves in high quantities in the environmental settings. More concerning are the gene profiles in biofilms from microplastics. The average gene copy number for ACC (8.94 ± 6.33 gene copies/16S rRNA), FOX (8.13 ± 2.46 gene copies/16S rRNA), and DHA (0.62 ± 0.46 gene copies/16S rRNA) were also a great concern, as it was observed that DHA presented low gene copy numbers in both microcosm samples of 2019 and river water samples in 2020 (Table 9 and 10).

Njage and Buys (2015) reported on the detection and transmission of AmpC β -lactamase genes by *E. coli* in water used for irrigating lettuce. It was highlighted that there is a possible high degree pathway for transmission of these genes in agricultural and food settings. AmpC β -lactamase gene groups have also been found in animal farms, where it was indicated that extended-spectrum β -lactamase and AmpC β -lactamases prevail in food animal production systems. Even though the antibiotic use profile differed, the results showed features indicating zoonotic transmission in the area (Lee *et al.*, 2020). Coertze and Bezuidenhout (2021), found a correlation between AmpC β -lactamases, highly populated areas, and bacterial family groups known to carry pathogenic species, including *Burkholderiaceae*, *Pseudomonadaceae*, *Aeromonadaceae*, and *Enterobacteriaceae*. In this study, high levels of *Enterobacteriaceae*, *Pseudomonadaceae* families, and genera such as *Aeromonas*, *Pedobacter*, *Citrobacter* were detected on both microplastics and river water samples from the urban areas and more pollution exposed sites than the pristine site. The bacterial species (pathogenic and non-pathogenic) belonging to families such as the *Enterobacteriaceae*, *Aeromonadaceae*, and *Pseudomonadaceae* can still exchange and transfer ARG between other bacterial groups. For example, *Pedobacter* is known to inhabit a diverse range of habitats (soil, water, glaciers, etc.), and are regarded as superbugs because they encode a large variety of resistance genes and mechanisms. They are also regarded as reservoirs of β -lactamases (Viana *et al.*, 2018). OTUs from genus *Pedobacter* were detected in microplastics and water samples in this study, and in some instances found to be higher on microplastics.

CHAPTER 6: CONCLUSION

Water availability in South Africa's freshwater systems is low, thus, most of the water is re-used (Bouwman *et al.*, 2018). The impact of water pollution results in the deterioration of freshwater systems, mainly because the old machinery and technologies applied in purification systems cannot combat and remove heavy loads of ARB, ARG, and microplastics. There is minimal research conducted on the detection and prevalence of microplastics in the river systems in Africa (Khan *et al.*, 2018). Of great concern is that microplastics can be a niche for a diverse array of microorganisms (pathogenic and non-pathogenic) that harbour ARG and may be dispersed to rural areas via the river system (Jin *et al.*, 2018). As a pilot simulation to understand the dynamics of microplastic-microorganism interactions, this study aimed to explore the behaviour of free-floating microorganisms when microplastics and wood substrates are introduced into the water medium through microcosm experiments for 14 days. The findings of the study showed that microorganisms colonized the substrates (microplastics and wood) within 48 hours of contact and biofilm formation in nutrient-rich water (i.e., WWTe) can occur as early as day 4. Furthermore, biofilms build-up takes place and premature biofilms can form within day 7 on microplastic substrates. Diversity indices showed that the BCC on microplastics was less rich, diverse, and less even than in water and wood substrates.

Following microcosm experiments, another objective of this study was to investigate the microplastic-microorganism interactions in the environment. In this study, it was observed that microplastic fibres are highly abundant throughout the water column. In the more urban-industrial sites there is a diverse array of microplastic types (fragments, pellets, foams, and films). Moreover, microplastics were colonized by eukaryotic and bacterial cells. Most of the surfaces of the microplastics were covered by biofilms of bacterial cells. The BCC on microplastics was also less rich and diverse, as observed in the microcosm experiments. However, field environmental microplastics were more even than the free-living microbial community in water. To understand the factors that may drive antibiotic resistance of clinically relevant microorganisms and ARG in the environment. This study investigated the prevalence and quantity of ARG, specifically AmpC β -lactamase gene groups and the *intl1* gene on microbial communities from microplastics in both microcosms and environmental samples, including the water samples. The results showed similarities with Coertze and Bezuidenhout's (2020) findings in terms of ARG; that is, the *intl1* gene and AmpC genes were prevalent in

water samples and microplastic samples isolated from highly polluted areas. The complete summary of the outcomes of the objectives of this study are discussed below:

6.1 Objective 1: To conduct microcosm studies to investigate biofilm formation on substrates visually using SEM.

It is well acknowledged that microorganisms can exist, attach, and build biofilms on particles. This is usually observed in stagnant materials such as rocks and water pipes (Battin *et al.*, 2016). To understand the relationship between microplastic-microorganism interactions in freshwater systems, this study incubated three types of sterile microplastics (LD-PE, PP, and PS) and wood (non-plastic control) substrates into surface running water and WWTe for 14 days. SEM images showed that microbial cells attached more to the rough surface parts (i.e., pits, folds, crevices) of the microplastics than the smooth area. Some of the cells are attached to the surface by using their cell extension and prosthecae to hold on to the surface. This indicated that the microplastic surface and some microbial cell surface plays a major role in the colonization and attachment of microorganisms on microplastics and wood substrates (Liao *et al.*, 2015). To further support this observation was the presence of the *Hyphomonadaceae* family which contains members with prosthecate and pili-like structures. With longer incubation, the study observed a build-up of biofilms on both microplastic and wood substrates. The results showed that the process occurred more rapidly in nutrient-rich water of WWTe than in pristine water. Oberbeckmann *et al.* (2018) made similar observations in the study. The results from this study serve as confirmation that the processes are the same even in marine settings.

In terms of substrate type, the LD-PE and PP used in this study were solid fragments and microbial colonization on these substrates was somewhat similar. The difference was that LD-PE polymers were smooth but presented with areas that had degradation, in which biofilms flourished (Figure 11 and 14). On PP, microbial cells were attached to the fold and pits of the substrates. The colonization process on PS foams was difficult to observe with SEM because microorganisms entered the deeper parts of the polymer type to form communities, whereas 16S metabarcoding confirmed the process of colonization. Wood had more microbial colonization in both river and WWTe microcosms, possibly due to the complex natural design structure of the particles. The results indicated that microplastics and wood are a niche for microbial communities and that the process of colonization by microbial cells is not hindered by turbulence. In environmental settings, as microplastics travel across the water column, they pick up other bacterial cells that are attached and may be transported to other parts of the river.

6.2 Objective 2: To characterize microbial consortia on microplastics in WWTe versus river water

Following microcosm experiments and SEM observations for microbial colonization, 16S metabarcoding analysis was used to identify the BCC and determine the distribution of the microbial groups on the substrates versus the surrounding water. The BCC was dominated by Proteobacteria and Bacteroidota phyla, which was expected because these phyla contain the largest number of microbial members and are regarded as common primary and secondary colonizers of microplastics (Yang *et al.*, 2020). The distribution and dominance of bacterial phyla differed between the microplastics, wood substrates, and water. Furthermore, BCC differed between the polymer types and source water (river water versus WWTe). As observed from the earliest day of colonization to the end of the experiment, the BCC on the substrates fluctuated composition and dominance leading to the introduction of other bacterial phyla. This would suggest that microbial cells may have attached opportunistically on the substrates, or either cell death occurred by microbial competition between the groups, or the substrate provided no beneficial use (Lavery *et al.*, 2020).

Microbial community profiling revealed that the microbial consortia on microplastics were less rich, diverse, and even than wood and the surrounding the free-living microbial community in water. In terms of polymer types, the microbial community on PP was richer and more diverse but less even than in LD-PE and PS. Biomarker analysis revealed that genera *Aeromonas*, *Acinetobacter*, and *Pseudomonas* were collectively enriched on microplastics and wood substrates than in the surrounding water column, with some bacterial taxa more enriched in the water community than on the substrates. Studies that have made similar observations suggest that microorganisms may be selective and only bacterial groups that benefit from microplastics (i.e., feed on the carbon source) may survive, and others may just be hitchhiking on the substrate (McCormick *et al.*, 2014; Bryant *et al.*, 2016; Miao *et al.*, 2019). We further observed that genera reported to degrade polymers such as *Ideonella* and *Acinetobacter* were more enriched on microplastics than wood and the surrounding water.

6.3 Objective 3: To characterize the microbial consortia on microplastics isolated from the river system

The study embarked on finding microplastics in the river system to characterize the microbial composition on the substrates to gain insight into what occurs in the natural environment. Microplastics were isolated from five sampling points along the Mooi River. Fibre pieces were the most dominant microplastics, followed by fragments, PS-foams, films, and microbeads (Figure 23). This is consistent with research studies that have isolated microplastics in the

aquatic systems (Bouwman *et al.*, 2018; Zhang *et al.*, 2019). Following the isolation of microplastics, the study investigated the surface of the substrates using SEM. The SEM images revealed that microplastics were aged and showed degradation and some microplastics originated from large pieces of plastics (Figure 22 and 24). All microplastics viewed under SEM were enriched with biofilm patches, and possible active cell growth on the surface was observed. Moreover, the images revealed that a symbiotic community exists between eukaryotic and bacterial cells (Zettler *et al.*, 2013).

Phyla distribution varied across the sites and between microplastic samples and the surrounding water microbial community from where the substrates were isolated from. These differences in BCC between sites and microplastics can be attributed to factors such as geographical location and the microbial colonization process (Oberbeckmann *et al.*, 2018). Unlike in the microcosms experiments where sterile substrates we introduced into the water medium, microplastics come from different sources in the natural environment. They are already colonized by different types of taxa from where they originate. Upon entering the water column, they may already be in the process of formulating premature biofilms. The BCC at the different sites was also affected by the surrounding anthropogenic activities. For example, the pristine area such as Site M1 showed less BCC on microplastics than the surrounding water compared to the urban and industrial impacted sites such as Site G3.

Microbial community profiling observed in the microcosm studies and other research studies (McCormick *et al.*, 2014) showed that microplastics were less rich and less diverse than in the surrounding water community. The only difference observed was that the microbial community on microplastics was slightly more even than in the surrounding water community. Biomarker analysis showed that some microorganisms prefer living on substrates in biofilm communities. We observed that the *Enterobacteriaceae* and *Legionellaceae* families and genera such as *Bacteroides*, *Pedobacter*, *Hirschia* were significantly higher on microplastics than the surrounding water. More worrisome genera such as *Citrobacter*, *Staphylococcus*, and *Escherichia* *Shigella* contain members that are routinely reported as deadly infectious groups in clinical settings. In this study, they were enriched more on microplastics than the surrounding water. Furthermore, these bacterial groups are also known to carry and show resistance to a diverse array of antibiotics. The fact that most of the ESKAPE pathogens were found in WWTe microplastics in Site W4 and the downstream Site T5 indicated that microplastics are not removed in WWTP, and they escape these processes carrying pathogenic organisms and may disperse them to further parts of the environment (McCormick *et al.*, 2014; Oberbeckmann *et al.*, 2018; Luo *et al.*, 2019).

6.4 Objective 4: To detect and quantify ARG from microplastic biofilms and water samples

To understand what drives and influences antibiotic resistance, surveillance of ARB and ARG in the environment is of fundamental importance. Currently, it is known that river systems, including WWTP are reservoirs for ARG and can disseminate ARG and ARB and pathogenic organisms to the receiving water body (Marti *et al.*, 2014). In South Africa, most treatment plants are mismanaged. Therefore, the quantities and levels of pathogenic organisms and ARG released into the receiving water body are a cause for concern considering that some rural areas depend on river water for domestic purposes (Oberholster and Ashton, 2008). Microplastics have been reported as new hotspots for the exchange of ARG in river systems. Arias-Andres *et al.* (2018a) reported a higher rate of HGT transfer within the plastsphere through microcosm studies, which is in assistance with mobile genetic elements. This study investigated the prevalence of ARG on microplastics versus water in both microcosm and field environmental samples.

The results from the microcosm indicated that bacterial communities carry and are responsible for the distribution of ARG within the plastsphere. The study revealed that in pristine water, there was less abundance of ARG than in polluted water. In river water, 1/6 AmpC gene group (FOX), as opposed to 3/6 AmpC gene groups (FOX, MOX/CMY, and DHA) were detected in WWTe substrates and water samples. Moreover, the *int1* gene was only detected in WWTe water and substrates. The WWTe is highly concentrated with influents from various sources and thus is a reservoir of ARG and ARB, which explains the diverse array of resistance genes. This was also a similar concept in the field environment results. In pristine environments such as Site M1, only 2/6 ARG we detected, whereas other sites contained more than 4/6 ARG, which were in association with the *int1* gene. The *int1* gene is usually detected in anthropogenically impacted sites (Gillings *et al.*, 2014). The Sites (O2, G3, W4, T5) where the AmpC genes groups and the *int1* gene were abundant and diverse are the sites that are impacted by various pollutants from the surroundings. The great concern is that ARG transfer is more frequent within biofilm communities between pathogenic and non-pathogenic organisms and ARG carriers.

Based on the prevalence and copy numbers of AmpC β -lactamase gene groups on microplastics and water samples in highly populated areas, the findings in this study confirmed the observations of Coertze and Bezuidenhout (Coertze and Bezuidenhout, 2020; Coertze and Bezuidenhout, 2021), which showed there is a link between AmpC genes and population density. Moreover, it was observed that taxa known to contain AmpC genes and are of clinical importance such as the *Enterobacteriaceae* family and genera *Citrobacter*, *Aeromonas*,

*Escherichia*_ *Shigella*, *Enterobacter*, including the environmental superbug, the *Pedobacter* genus, may have been the influence for the presence of AmpC genes. However, these require further investigation, and culturing techniques would need to be applied to determine copy numbers within the isolated groups. What this study found is that microplastics are a habitat for microorganisms, and based on the substrate type, the microbial consortia are unique. Furthermore, in environmental settings, these polymers can enhance and facilitate the transport and transfer of ARG between microplastic-microorganism communities and the free-living bacterial taxa found in the surrounding water.

6.5 Recommendations

The findings from this research provided some insights into the microplastic-microorganism interactions in river settings from South Africa's context. However, more research is still required into understanding these interactions. The following recommendations are proposed for future studies:

future studies:

- Research studies focusing on formulating a standardized method of microplastic identification and isolation from freshwater systems should be conducted. This would assist in setting a baseline for understanding the burden of microplastics in South Africa's aquatic systems.
- Microorganisms known to degrade microplastics should be investigated. Also, their enzymes and the rate of degradation on different polymers should be conducted to assist in the formulation of mitigation plans for irradiating microplastics in freshwater and marine settings.
- This study served as a pilot study for investigating the BCC on microplastics in South Africa, as thus sampling sites were limited. It is proposed that more sampling be done in South Africa freshwater systems. Moreover, studies should also investigate the relationship between microplastics ingested by aquatic animals and the adverse effects pathogens have on these organisms. This would provide a picture of the extent of microplastic pollution, the relationship between microplastics and pathogenic organisms, and their health impact on aquatic biota.
- This study observed that WWTP serve as an escape for microplastics and are carrying with them ARG through biofilms. Future studies should investigate and formulate methods that may target the removal of microplastics from effluent and destroy biofilm communities on microplastics.
- More ARG relating to antibiotic usage in South Africa should be screened for, in microplastic biofilm studies. This would assist the studies in observing the prevalence of ARG in the aquatic systems and what drives and disperse ARG throughout the water column in downstream parts that are not impacted by WWTP.

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ANNEXURES

Table S1 A: Distribution of biomarkers detected on microplastics and from the river water microcosm vessel at genus level determined using LEfSe biomarker analysis

Genus	P-value	Microplastic	Water	Wood	LDA score
<i>Romboutsia</i>	0.041	0	0	20155	4
<i>Flavobacterium</i>	0.058	1182100	1659100	184130	5.87
<i>TM7a</i>	0.072	0	45455	41400	4.36
<i>WD2101_soil_group</i>	0.088	2014.4	45455	23050	4.34
<i>Lacihabitans</i>	0.114	10738	130680	6772	4.79
<i>Flectobacillus</i>	0.121	28000	51136	0	4.41
<i>Sphingomonas</i>	0.124	18650	573860	728050	5.55
<i>Emticicia</i>	0.131	3532.3	107950	4582	4.72
<i>Roseomonas</i>	0.138	3700.3	0	136060	4.83
<i>Prostheco bacter</i>	0.149	6958.9	181820	10930	4.94
<i>Aquabacterium</i>	0.194	486920	0	2364.9	5.39
<i>Bdellovibrio</i>	0.205	7691.5	0	26888	4.13
<i>Hyphomicrobium</i>	0.207	12075	107950	24133	4.68
<i>Hydrogenophaga</i>	0.212	123900	619320	204050	5.39
<i>Dechloromonas</i>	0.233	90490	511360	193380	5.32
<i>Cellvibrio</i>	0.238	43035	278410	59449	5.07
<i>Saccharimonadales</i>	0.266	25865	0	131210	4.82
<i>Sediminibacterium</i>	0.266	17318	0	245360	5.09
<i>Pedobacter</i>	0.266	15112	0	58484	4.47
<i>R7C24</i>	0.273	637080	164770	23353	5.49
<i>Duganella</i>	0.274	19498	90909	13303	4.59
<i>Fluviicola</i>	0.274	31682	102270	629.79	4.71

<i>Rhodobacter</i>	0.284	104670	1079500	81500	5.7
<i>Chryseobacterium</i>	0.288	11586	0	10149	3.76
<i>Methylobacter</i>	0.295	47296	147730	13118	4.83
<i>Lysobacter</i>	0.341	1233.4	0	85742	4.63
<i>Gemmobacter</i>	0.341	40341	0	169650	4.93
<i>Brevundimonas</i>	0.341	2466.9	0	145370	4.86
<i>Acinetobacter</i>	0.347	904190	0	174220	5.66
<i>Dyadobacter</i>	0.349	7629.7	34091	45327	4.28
<i>Haliscomenobacter</i>	0.360	24653	51136	4966.1	4.36
<i>Acidovorax</i>	0.422	351350	0	171790	5.24
<i>Novosphingobium</i>	0.426	347660	0	517690	5.41
<i>uncultured</i>	0.448	603290	2204500	857720	5.9
<i>Rhodoferax</i>	0.461	8489	0	46204	4.36
<i>Phaeodactylibacter</i>	0.461	1648.2	0	6106.5	3.48
<i>Perlucidibaca</i>	0.461	17102	0	917530	5.66
<i>OM190</i>	0.461	6775.8	0	39280	4.29
<i>Aeromonas</i>	0.461	436990	0	210580	5.34
<i>Hirschia</i>	0.490	285520	0	22076	5.15
<i>Nocardioides</i>	0.509	62683	204550	136520	4.85
<i>Sphingobium</i>	0.551	245460	0	35543	5.09
<i>Lactobacillus</i>	0.563	595160	0	45505	5.47
<i>Sphingopyxis</i>	0.569	312600	0	13535	5.19
<i>Bosea</i>	0.585	18657	0	16320	3.97
<i>Lautropia</i>	0.585	4395.1	0	4379.9	3.34
<i>Lacunisphaera</i>	0.585	58548	0	10402	4.47
<i>Sphaerotilus</i>	0.585	17558	0	22873	4.06
<i>Brevifollis</i>	0.585	15932	0	17753	3.95

<i>Zoogloea</i>	0.589	13010	0	49657	4.39
<i>Arenimonas</i>	0.599	38696	0	16604	4.29
<i>Pseudorhodobacter</i>	0.601	74400	0	29638	4.57
<i>Allorhizobium_Neorhizobium_</i>					
<i>Pararhizobium_Rhizobium</i>	0.630	32132	0	20522	4.21
<i>Paracoccus</i>	0.632	228090	346590	852420	5.49
<i>Terrimonas</i>	0.676	7981.4	0	36788	4.26
<i>Rubellimicrobium</i>	0.676	3465.5	0	5242.6	3.42
<i>Reyranella</i>	0.704	35462	0	112870	4.75
<i>Sphingorhabdus</i>	0.728	241700	0	7833.7	5.08
<i>SM1A02</i>	0.737	6209	0	5830.8	3.49
<i>Pirellula</i>	0.737	62193	0	24391	4.49
<i>Methylobacterium_Methylorubrum</i>	0.737	80662	0	45541	4.61
<i>Limnohabitans</i>	0.737	7977.2	0	6408.4	3.6
<i>Vogesella</i>	0.737	138480	0	95471	4.84
<i>Rheinheimera</i>	0.740	398590	193180	98719	5.18
<i>Stenotrophobacter</i>	0.764	21961	0	8134.8	4.04
<i>Massilia</i>	0.803	227920	0	159830	5.06
<i>Mesorhizobium</i>	0.803	6547.7	0	5466	3.52
<i>OLB12</i>	0.803	332370	272730	161180	4.93
<i>Babeliales</i>	0.803	20854	0	116560	4.77
<i>SWB02</i>	0.833	48955	0	53020	4.42
<i>Pseudomonas</i>	0.927	257980	272730	698840	5.34

Table S1 B: Distribution genera detected on microplastics and wood from the WWTe microcosm analyzed at genus level determined using LEfSe biomarker analysis

Genus	P value	Microplastic	WWTE	Wood	LDA score
<i>Bosea</i>	0.053	0	16130	12440	3.91
<i>Ferruginibacter</i>	0.053	0	31893	13986	4.2
<i>NS9_marine_group</i>	0.053	0	135710	9719.5	4.83
<i>Enterococcus</i>	0.053	0	586.54	9215.3	3.66
<i>Roseomonas</i>	0.053	0	7478.3	2296	3.57
<i>Blastomonas</i>	0.065	0	4692.3	15318	3.88
<i>Silanimonas</i>	0.065	0	8504.8	6422.2	3.63
<i>Caulobacter</i>	0.065	0	37172	32325	4.27
<i>Hydrogenophaga</i>	0.090	301180	648050	76413	5.46
<i>Duganella</i>	0.098	7179.6	0	102950	4.71
<i>Dechlorobacter</i>	0.105	0	6012	1368.2	3.48
<i>Flaviumibacter</i>	0.105	0	12757	3154.1	3.8
<i>Crocinitomix</i>	0.105	0	17449	3154.1	3.94
<i>Aurantisolimonas</i>	0.105	0	22875	228.04	4.06
<i>Blfdi19</i>	0.105	0	27934	2651	4.15
<i>Enterobacter</i>	0.105	0	9311.3	1311.2	3.67
<i>Escherichia_Shigella</i>	0.105	0	9751.2	1026.2	3.69
<i>Candidatus_Ovatusbacter</i>	0.105	0	1906.2	541.59	2.98
<i>Comamonas</i>	0.105	0	1686.3	826.64	2.93
<i>WPS_2</i>	0.105	0	29987	6571	4.18
<i>Ilumatobacter</i>	0.105	0	1539.7	456.08	2.89
<i>Lautropia</i>	0.105	0	29473	4468.3	4.17
<i>LiUU_11_161</i>	0.105	0	659.85	285.05	2.52
<i>Haliscomenobacter</i>	0.105	0	6451.9	1026.2	3.51
<i>Pir4_lineage</i>	0.105	0	26321	769.63	4.12
<i>Peredibacter</i>	0.105	0	42231	370,56	4.32
<i>Runella</i>	0.105	0	65179	1368.2	4.51

<i>Pedobacter</i>	0.137	13611	91866	39908	4.59
<i>Dyadobacter</i>	0.137	1047	22362	6935.3	4.03
<i>SWB02</i>	0.153	972.24	43257	0	4.34
<i>Phreatobacter</i>	0.153	1421	14150	0	3.85
<i>Oikopleura</i>	0.153	5097.7	35119	0	4.24
<i>Coxiella</i>	0.153	822.66	10191	0	3.71
<i>Methylophilus</i>	0.172	3398.3	28080	0	4.15
<i>Pseudoxanthomonas</i>	0.182	8496.2	47289	59332	4.41
<i>IMCC26134</i>	0.189	0	1979.6	3164.1	3.2
<i>Asticcacaulis</i>	0.189	0	659.85	5159.4	3.41
<i>Fluviimonas</i>	0.189	0	4399	5815	3.46
<i>Romboutsia</i>	0.189	0	2492.8	19187	3.98
<i>Nitrosomonas</i>	0.189	0	659.85	2365.6	3.07
<i>67_14</i>	0.189	0	1686.3	2365.6	3.07
<i>DEV007</i>	0.189	0	14443	16559	3.92
<i>Mesorhizobium</i>	0.189	0	1613	6571	3.52
<i>Altererythrobacter</i>	0.191	22218	597530	3933.7	5.47
<i>Novosphingobium</i>	0.206	36896	176470	1126800	5.74
<i>Brevundimonas</i>	0.211	8496.2	35339	13888	4.13
<i>Paucibacter</i>	0.211	11069	90180	8480,8	4.61
<i>OLB12</i>	0.223	236310	729360	0	5.56
<i>Prostheco bacter</i>	0.223	33360	66059	0	4.52
<i>Methylotenera</i>	0.248	1154.7	81309	57.01	4.61
<i>Phaselicystis</i>	0.248	21586	95752	5256.8	4.66
<i>Lysobacter</i>	0.248	822.66	6965.1	85.515	3.54
<i>Terrimonas</i>	0.248	8071.4	33506	3905.2	4.17
<i>Porphyrobacter</i>	0.248	6796.9	52788	6833.8	4.36
<i>Reyranella</i>	0.248	1271.4	30720	2052.4	4.17
<i>Candidatus_Peregrinibacteria</i>	0.248	1047	19649	525.68	3.98
<i>Arcicella</i>	0.250	1667.9	4399	83503	4.61

<i>Pirellula</i>	0.273	5743.8	241730	2850,5	5.08
<i>Aquabacterium</i>	0.300	23914	18696	82756	4.51
<i>Acinetobacter</i>	0.300	213600	87541	1956500	5.97
<i>Pseudomonas</i>	0.300	390490	170760	428520	5.11
<i>Cellvibrio</i>	0.312	97046	199130	8180,9	4.98
<i>Rhodoferax</i>	0.325	67972	215990	55742	4.9
<i>Sphingopyxis</i>	0.325	65937	354560	20857	5.22
<i>CL500_29_marine_group</i>	0.344	1667.9	10338	5519.6	3.64
<i>Gemmobacter</i>	0.344	5388.5	36439	222480	5.04
<i>Emticicia</i>	0.355	10629	35119	26670	4.09
<i>Exiguobacterium</i>	0.364	15185	0	192070	4.98
<i>R7C24</i>	0.368	141290	133000	0	4.85
<i>Pseudorhodoplanes</i>	0.368	1794.9	1319.7	0	2.95
<i>Xanthobacter</i>	0.368	1421	3812.5	0	3.28
<i>Pseudohongiella</i>	0.368	3079.1	1173.1	0	3.19
<i>Cereibacter</i>	0.368	2991.5	3592.5	0	3.25
<i>Azospirillum</i>	0.368	1346.2	1759.6	0	2.94
<i>Phenylobacterium</i>	0.368	8496.2	1759.6	0	3.63
<i>Curvibacter</i>	0.368	11443	5938.7	0	3.76
<i>TRA3_20</i>	0.368	747.88	1613	0	2.91
<i>Lacunisphaera</i>	0.368	17661	14737	0	3.95
<i>Phaeodactylibacter</i>	0.368	8071.4	3079.3	0	3.61
<i>Perlucidibaca</i>	0.368	653450	12097	0	5.51
<i>Zoogloea</i>	0.385	15293	0	10016	3.88
<i>Hirschia</i>	0.392	115090	357490	5823.9	5.25
<i>uncultured</i>	0.392	497330	653840	338500	5.2
<i>Rheinheimera</i>	0.392	1651400	137910	464360	5.88
<i>GKS98_freshwater_group</i>	0.417	9300,6	0	0	3.67

Table S1-C: Distribution of OTUs on different substrates (LD-PE, PP, PS, and wood) versus river water at genus level determined using LEfSe biomarker analysis.

Genus	P value	River water	LD-PE	PP	PS	Wood	LDA score
<i>Flectobacillus</i>	0.094	51136	8489	0	75511	0	4.58
<i>Haliscomenobacter</i>	0.104	51136	73958	0	0	4966.1	4.57
<i>Flavobacterium</i>	0.156	1659100	1347400	636240	1564700	193950	5.86
<i>Lactobacillus</i>	0.163	0	0	1663000	122510	45505	5.92
<i>Not_Assigned</i>	0.166	261360	308430	0	704750	964610	5.68
<i>JG30_KF_CM45</i>	0.173	0	0	0	0	6176.3	3.49
<i>Romboutsia</i>	0.173	0	0	0	0	20155	4
<i>Pedobacter</i>	0.181	0	0	0	45336	58484	4.47
<i>WD2101_soil_group</i>	0.182	107950	0	0	6043.3	33700	4.73
<i>Lacihabitans</i>	0.210	130680	32215	0	0	6772	4.82
<i>Cellvibrio</i>	0.216	278410	109330	0	19778	59449	5.14
<i>Emticicia</i>	0.236	107950	0	0	10597	4582	4.73
<i>Aquabacterium</i>	0.248	0	18719	181900	1260100	2364.9	5.8
<i>Prostheco bacter</i>	0.260	181820	0	0	30216	17759	4.96
<i>Sphingomonas</i>	0.261	573860	0	0	55951	728050	5.56
<i>TM7a</i>	0.261	45455	0	0	0	41400	4.36

<i>Vicinamibacteraceae</i>	0.261	107950	0	0	0	50220	4.73
<i>Sphingobium</i>	0.265	0	659660	0	76729	35543	5.52
<i>Roseomonas</i>	0.290	0	11101	0	0	136060	4.83
<i>Duganella</i>	0.324	90909	0	0	58494	13303	4.66
<i>Fluviicola</i>	0.324	102270	0	0	95045	629.79	4.71
<i>Bdellovibrio</i>	0.327	0	0	0	23074	26888	4.13
<i>Rhodobacter</i>	0.332	1079500	129510	162520	21976	81500	5.72
<i>Chryseobacterium</i>	0.345	0	0	0	34758	10149	4.24
<i>Novosphingobium</i>	0.387	0	834780	181900	26280	517690	5.62
<i>Hyphomicrobium</i>	0.395	107950	0	27985	8240.9	24133	4.73
<i>Methylothermobacter</i>	0.403	147730	0	107280	34612	13118	4.87
<i>Noviherbaspirillum</i>	0.427	90909	2394.3	0	0	127680	4.81
<i>Sediminibacterium</i>	0.435	0	1959	0	49995	245360	5.09
<i>Saccharimonadales</i>	0.435	0	0	74627	2967.1	131210	4.82
<i>Dyadobacter</i>	0.468	34091	0	0	22889	45327	4.36
<i>Hydrogenophaga</i>	0.504	619320	91202	186570	93946	204050	5.42
<i>Acidovorax</i>	0.505	0	545770	473100	35181	171790	5.44
<i>Zoogloea</i>	0.509	0	8053.6	0	37725	158410	4.9
<i>R7C24</i>	0.509	164770	1205700	97948	607630	23353	5.77
<i>Dechloromonas</i>	0.533	511360	21331	231910	18227	193380	5.39

<i>Lautropia</i>	0.534	0	0	0	13185	4379.9	3.82
<i>Aeromicrobium</i>	0.534	0	3482.7	0	0	11587	3.76
<i>Lacunisphaera</i>	0.534	0	175640	0	0	10402	4.94
<i>Brevundimonas</i>	0.534	0	7400.6	0	0	145370	4.86
<i>Gemmobacter</i>	0.534	0	121020	0	0	169650	4.93
<i>Brevifollis</i>	0.534	0	0	0	47797	17753	4.38
<i>Bosea</i>	0.534	0	0	55970	0	16320	4.45
<i>Bacillus</i>	0.534	0	21549	0	0	5887.7	4.03
<i>Lysobacter</i>	0.534	0	3700.3	0	0	85742	4.63
<i>Sphaerotilus</i>	0.534	0	52675	0	0	22873	4.42
<i>Perlucidibaca</i>	0.552	0	0	51306	0	917530	5.66
<i>Rhodoferax</i>	0.552	0	25467	0	0	46204	4.36
<i>Bryobacter</i>	0.552	0	0	0	8240.9	6158.7	3.62
<i>Phaeodactylibacter</i>	0.552	0	0	0	4944.5	6106.5	3.48
<i>Aeromonas</i>	0.564	0	317140	955680	38145	210580	5.68
<i>Nocardioides</i>	0.569	204550	17849	158110	12087	136520	4.98
<i>Arenimonas</i>	0.589	0	0	88619	27470	16604	4.65
<i>uncultured</i>	0.604	2204500	846610	373130	606050	870840	5.96
<i>Hydrogenedensaceae</i>	0.656	0	52693	0	67026	2393.2	4.53
<i>Sphingorhabdus</i>	0.656	0	0	541040	184050	7833.7	5.43

<i>Acinetobacter</i>	0.672	0	495190	506630	1710700	174220	5.93
<i>Paracoccus</i>	0.672	346590	433810	250460	0	852420	5.63
<i>Rubellimicrobium</i>	0.683	0	4353.3	0	6043.3	5242.6	3.48
<i>Stenotrophobacter</i>	0.683	0	0	37313	28568	8134.8	4.27
<i>Pirellula</i>	0.699	0	0	177240	9339.6	24391	4.95
<i>Methylobacterium_Methylorubrum</i>	0.699	0	179360	0	62630	45541	4.95
<i>Vogesella</i>	0.699	0	90767	324680	0	95471	5.21
<i>Limnohabitans</i>	0.699	0	15454	0	8477.4	6408.4	3.89
<i>Mesorhizobium</i>	0.723	0	2612	0	17031	5466	3.93
<i>Abditibacterium</i>	0.723	0	4353.3	0	10438	4246	3.72
<i>Terrimonas</i>	0.728	0	12407	0	11537	36788	4.26
<i>Hirschia</i>	0.743	0	343710	242540	270300	22076	5.24
<i>Sphingopyxis</i>	0.768	0	2829.7	769590	165370	13535	5.59
<i>Rheinheimera</i>	0.771	193180	411170	659150	125470	98719	5.45
<i>Babeliales</i>	0.804	0	0	55970	6592.7	116560	4.77
<i>SWB02</i>	0.804	0	0	79291	67575	53020	4.6
<i>Pseudomonas</i>	0.884	272730	205040	256530	312360	698840	5.39
<i>OLB12</i>	0.891	272730	652490	97948	246680	161180	5.44
<i>Reyranella</i>	0.935	0	4571	79291	22525	112870	4.75

Table S1-D: Distribution of biomarkers on different substrates (LD-PE, PP, PS, and wood) versus WWTe at genus level determined using LEfSe biomarker analysis.

Genus	P value	WWTe	LD-PE	PP	PS	Wood	LDA score
<i>Roseomonas</i>	0.063	7047.1	0	0	0	2287.1	3.55
<i>NS9_marine_group</i>	0.063	127880	0	0	0	9713.3	4.81
<i>Ferruginibacter</i>	0.063	30054	0	0	0	14014	4.18
<i>Bosea</i>	0.063	15200	0	0	0	12416	3.88
<i>Enterococcus</i>	0.063	552.71	0	0	0	9166.4	3.66
<i>Silanimonas</i>	0.070	8014.3	0	0	0	6385.4	3.6
<i>Blastomonas</i>	0.070	4421.7	0	0	0	15233	3.88
<i>Rheinheimera</i>	0.137	132030	112420	2502200	94930	463610	6.08
<i>Novosphingobium</i>	0.142	166300	153260	14936	17011	1131500	5.75
<i>WPS_2</i>	0.150	28257	0	0	0	6532.2	4.15
<i>Ilumatobacter</i>	0.150	1450,9	0	0	0	458.06	2.86
<i>Peredibacter</i>	0.150	39795	0	0	0	372.18	4.3
<i>Runella</i>	0.150	124770	0	0	0	1546	4.8
<i>Flaviumibacter</i>	0.150	12021	0	0	0	3135.5	3.78
<i>Candidatus_Ovatusbacter</i>	0.150	1796.3	0	0	0	543.95	2.95

<i>Crocinitomix</i>	0.150	16443	0	0	0	3135.5	3.92
<i>Hydrogenedensaceae</i>	0.150	202090	50963	0	0	0	5
<i>Dechlorobacter</i>	0.150	5665.3	0	0	0	1374.2	3.45
<i>Aurantisolimonas</i>	0.150	21556	0	0	0	229.03	4.03
<i>Escherichia_Shigella</i>	0.150	9188.8	0	0	0	1030,6	3.66
<i>Pseudoxanthomonas</i>	0.164	44562	0	11263	0	58986	4.47
<i>Pir4_lineage</i>	0.189	24803	0	2199.5	0	2602	4.09
<i>Phreatobacter</i>	0.199	13334	0	1899.6	0	0	3.82
<i>Oikopleura</i>	0.199	33094	0	6757.9	0	0	4.22
<i>Kaistia</i>	0.199	1934.5	0	599.86	0	0	2.99
<i>Exiguobacterium</i>	0.199	0	0	20167	0	190950	4.98
<i>Pseudohongiella</i>	0.228	1105.4	6159.8	0	0	0	3.49
<i>Romboutsia</i>	0.228	2349	0	0	0	19074	3.98
<i>Asticcacaulis</i>	0.228	621.8	0	0	0	5181.9	3.41
<i>Mesorhizobium</i>	0.228	1520	0	0	0	6532.2	3.51
<i>Phaselicystis</i>	0.228	82769	0	0	42735	0	4.62
<i>Comamonas</i>	0.228	5388.9	0	0	13169	0	3.82
<i>Fluviimonas</i>	0.228	4145.3	0	0	0	5840,3	3.47
<i>Perlucidibaca</i>	0.228	11400	0	0	1293700	0	5.81
<i>Bacteroides</i>	0.228	414.53	0	0	0	1829	2.96

<i>Legionella</i>	0.228	2072.7	0	0	0	1829	3.02
<i>Chloroplast</i>	0.229	0	68785	147620	0	6556	4.87
<i>DEV007</i>	0.230	192140	1540	0	0	16461	4.98
<i>Bryobacter</i>	0.232	138.18	0	5641.7	0	0	3.45
<i>Vicinamibacteraceae</i>	0.232	2279.9	0	46236	0	0	4.36
<i>Hyphomicrobium</i>	0.232	1105.4	0	26315	0	0	4.12
<i>Duganella</i>	0.240	0	0	9597.8	37862	102500	4.71
<i>Pedobacter</i>	0.251	86568	0	20495	20577	39715	4.64
<i>Lacihabitans</i>	0.259	124430	116330	24426	0	85315	4.79
<i>Rhodoferax</i>	0.268	203540	35676	66765	0	55942	5.01
<i>Deinococcus</i>	0.269	0	0	9947	0	0	3.7
<i>Agromyces</i>	0.269	0	0	6594.6	0	0	3.52
<i>Candidatus_Peregrinibacteria</i>	0.270	18792	0	1399.7	0	3396.7	3.97
<i>Cellvibrio</i>	0.274	187650	117570	114600	0	8216.5	4.97
<i>Rhodobacter</i>	0,275	147230	59032	443310	0	136360	5.35
<i>Brevundimonas</i>	0.284	33301	0	11263	0	13897	4.22
<i>Thermomonas</i>	0.284	25287	0	39421	0	14918	4.29
<i>Porphyrobacter</i>	0.295	49744	0	9010,5	0	6793.5	4.4
<i>Reyranella</i>	0.295	28948	0	1699.6	0	2061.3	4.16
<i>Lysobacter</i>	0.295	6563.4	0	1099.7	0	85.887	3.52

<i>Altererythrobacter</i>	0.300	563070	5133.2	26119	0	3950,8	5.45
<i>Paucibacter</i>	0.311	84979	0	14797	2469.3	8450,3	4.63
<i>Bradyrhizobium</i>	0.313	5112.6	0	14962	0	916.13	3.87
<i>Sphingopyxis</i>	0.316	334110	0	23710	95183	20805	5.22
<i>Sphingobium</i>	0.316	553750	638560	19671	445890	45020	5.49
<i>Ideonella</i>	0.319	9534.3	0	0	161230	687.1	4.91
<i>Ahniella</i>	0.319	35788	0	0	33023	4964.5	4.25
<i>Haliscomenobacter</i>	0.319	6079.8	67950	0	0	1030,6	4.53
<i>Haliangium</i>	0.340	4559.9	0	4505.3	0	0	3.36
<i>Azospirillum</i>	0.340	1658.1	0	1799.6	0	0	2.95
<i>SH3_11</i>	0.340	1865.4	0	7321.1	0	0	3.56
<i>Not_Assigned</i>	0.346	571780	159390	450610	165990	927600	5.58
<i>Mycobacterium</i>	0.411	8774.3	0	0	13598	15677	3.89
<i>Clostridium_sensu_stricto_1</i>	0.411	1036.3	9496.4	0	0	7054.8	3.68
<i>Subgroup_17</i>	0.411	552.71	0	0	548.73	2612.9	3.12
<i>Methylophilus</i>	0.411	26461	2309.9	2999.3	4389.8	0	4.12
<i>Methylotenera</i>	0.411	85808	2309.9	3942.1	0	57.258	4.63
<i>Sphingomonas</i>	0.426	0	0	87264	36216	0	4.64
<i>Sericytochromatia</i>	0.446	4767.1	0	6194.7	0	229.03	3.49
<i>Candidatus_Campbellbacteria</i>	0.446	4836.2	0	2499.4	0	2090,3	3.38

<i>Hirschia</i>	0.486	336880	334070	17458	196190	5808.8	5.22
<i>Shewanella</i>	0.495	0	0	0	4115.5	2090,3	3.31
<i>Zoogloea</i>	0.569	0	0	20274	24418	9957.6	4.09
<i>Massilia</i>	0.570	483.62	0	243370	480680	100340	5.38
<i>Acinetobacter</i>	0.587	82492	53129	471290	1411900	1948900	5.98
<i>Hydrogenophaga</i>	0.606	610680	100610	165160	250580	76554	5.43
<i>Aeromonas</i>	0.607	15130	6416.5	531000	118150	157560	5.42
<i>Trichococcus</i>	0.620	0	0	9573.7	0	19335	3.99
<i>Pseudomonas</i>	0.636	160910	166320	259250	389650	427530	5.12
<i>Lactobacillus</i>	0.637	967.24	0	600360	42735	2862.9	5.48
<i>Actibacter</i>	0.687	0	0	1499.7	0	257.66	2.88
<i>Ferribacterium</i>	0.687	0	0	1799.6	0	343.55	2.95
<i>Rhizobacter</i>	0.687	0	0	9573.7	0	6584.7	3.68
<i>Clade_III</i>	0.687	0	1796.6	7398.3	0	0	3.57
<i>Lacibacter</i>	0.687	0	0	14079	0	5067.3	3.85
<i>Acetobacter</i>	0.687	0	0	441400	50505	0	5.34
<i>Flavobacterium</i>	0.896	1336900	1290400	834910	1450700	1524000	5.54
<i>Limnohabitans</i>	0.901	2072.7	1064900	140370	5487.3	34489	5.73
<i>Flectobacillus</i>	0.904	6632.5	2823.3	14689	1033000	6928.2	5.71

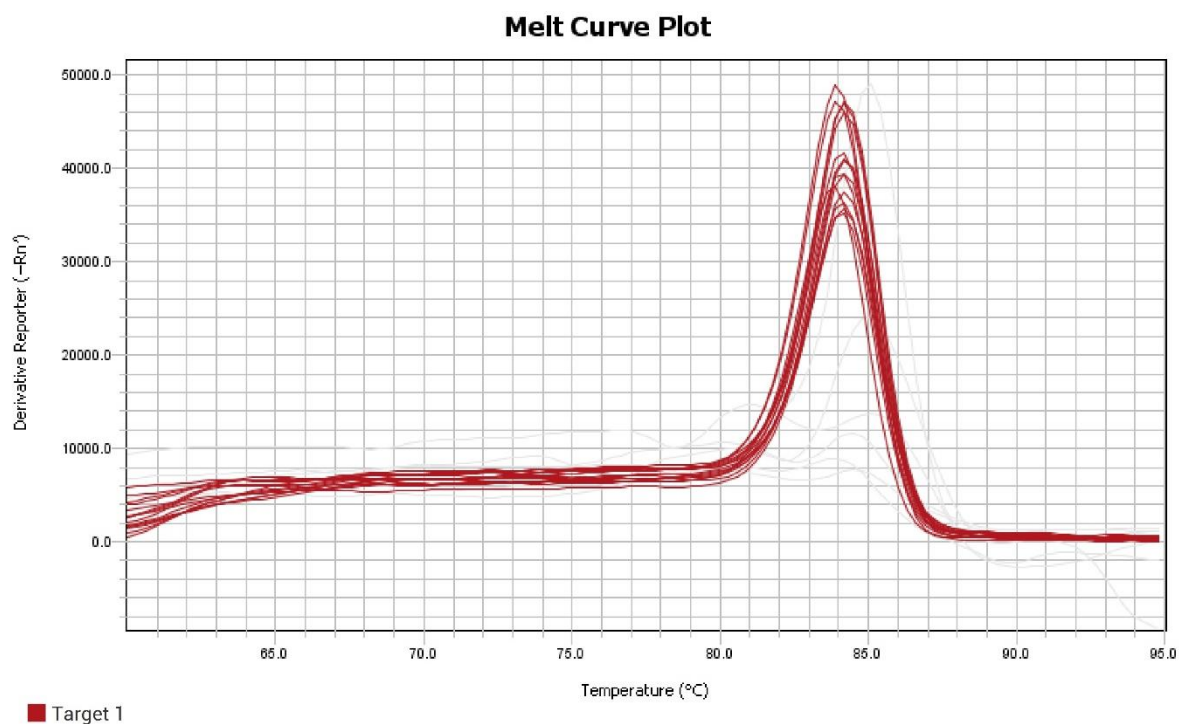


Figure S1-A: Melt curve plot for the 16S rRNA gene standards

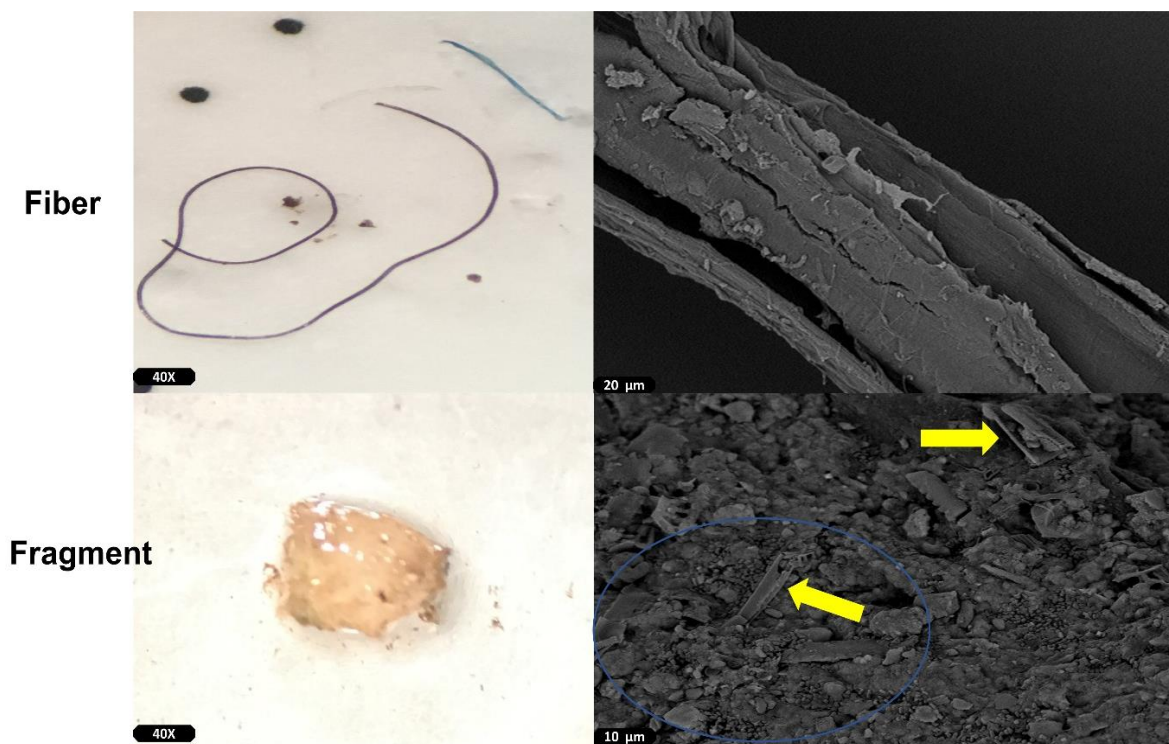


Figure S1-B: Stereomicroscope and SEM images showing examples of the surfaces of the microplastic fibre and fragment covered with biofilms made of diverse microbial community.

Table S2-A: Bacterial taxa abundance on microplastics versus water from the Mooi River.

Genus	P-values	microplastics	water	LDA score
<i>Exiguobacterium</i>	0.001	2094.4	224420	5.05
<i>Legionella</i>	0.024	14697	0	-3.87
<i>Pedobacter</i>	0.034	30230	765.68	-4.17
<i>Escherichia_Shigella</i>	0.039	27291	0	-4.14
<i>Hydrogenedensaceae</i>	0.050	19725	260.55	-3.99
<i>Rheinheimera</i>	0.051	330670	798230	5.37
<i>Hirschia</i>	0.057	48758	2541.7	-4.36
<i>Gemmata</i>	0.061	13484	0	-3.83
<i>alpha_cluster</i>	0.061	3934.9	0	-3.29
<i>Bacteroides</i>	0.061	17497	0	-3.94
<i>Methyloversatilis</i>	0.061	2148.5	0	-3.03
<i>Macellibacteroides</i>	0.061	9611.2	0	-3.68
<i>Massilia</i>	0.063	33755	81005	4.37
<i>Citrobacter</i>	0.092	7872.6	0	-3.6
<i>Desulfovibrio</i>	0.092	1727.1	0	-2.94
<i>Propionivibrio</i>	0.092	1289.7	0	-2.81
<i>Rubellimicrobium</i>	0.092	117630	0	-4.77
<i>OM190</i>	0.092	3311.9	0	-3.22
<i>Geminicoccus</i>	0.092	3276	0	-3.21
<i>R7C24</i>	0.124	61415	6774.4	-4.44
<i>OM27_clade</i>	0.133	41166	1823.9	-4.29
<i>Flectobacillus</i>	0.133	11837	70123	4.46
<i>Shewanella</i>	0.134	972.17	5999.5	3.4
<i>Streptococcus</i>	0.135	12094	0	-3.78
<i>Xanthobacter</i>	0.135	8854.4	0	-3.65
<i>Proteocatella</i>	0.135	7838	0	-3.59
<i>Kosakonia</i>	0.135	3158.6	0	-3.2
<i>Saccharofermentans</i>	0.135	1135.5	0	-2.75

<i>Craurococcus_Caldovatus</i>	0.135	1881.5	0	-2.97
<i>AB1</i>	0.135	7117.5	0	-3.55
<i>Azospirillum</i>	0.135	17592	0	-3.94
<i>PeM15</i>	0.135	7199.7	0	-3.56
<i>Cellulomonas</i>	0.135	5971	0	-3.48
<i>Blastomonas</i>	0.135	2076	0	-3.02
<i>Enhydrobacter</i>	0.135	747.64	0	-2.57
<i>Aeromicrobium</i>	0.135	20419	0	-4.01
<i>Paucibacter</i>	0.153	106.16	1266	2.76
<i>Gemmobacter</i>	0.161	236440	15953	-5.04
<i>Sphingobium</i>	0.188	34862	8208.5	-4.12
<i>Blfdi19</i>	0.193	3705.1	781.66	-3.17
<i>Mycobacterium</i>	0.194	23577	3963	-3.99
<i>Rivicola</i>	0.194	1664	0	-2.92
<i>Aquaspirillum_polymorphum_group</i>	0.194	2948.5	0	-3.17
<i>Flexithrix</i>	0.194	54326	0	-4.43
<i>Pseudoxanthobacter</i>	0.194	96245	0	-4.68
<i>Kaistia</i>	0.194	2942.9	0	-3.17
<i>Chitinivorax</i>	0.194	10531	0	-3.72
<i>SAR324_clade (Marine_group_B)</i>	0.194	1360.8	0	-2.83
<i>Blastococcus</i>	0.194	5748.4	0	-3.46
<i>Polaromonas</i>	0.194	5322	0	-3.43
<i>Cetobacterium</i>	0.194	3127.2	0	-3.19
<i>Geodermatophilus</i>	0.194	1768.9	0	-2.95
<i>C2U</i>	0.194	6432.7	0	-3.51
<i>Rhodobacter</i>	0.211	253400	282520	4.16
<i>Caulobacter</i>	0.216	11270	1445.9	-3.69
<i>Steroidobacter</i>	0.217	1729.8	2909.4	2.77
<i>Hydrogenophaga</i>	0.228	208670	107510	-4.7
<i>Brevundimonas</i>	0.229	343060	8374.7	-5.22

<i>Enterobacter</i>	0.239	10266	324.92	-3.7
<i>Reyranella</i>	0.239	43117	1667.7	-4.32
<i>Sphingopyxis</i>	0.240	117640	28009	-4.65
<i>Methylobacterium_Methylorubrum</i>	0.257	132560	34002	-4.69
<i>Lactobacillus</i>	0.257	27859	5753.6	-4.04
<i>Pirellula</i>	0.262	45754	58506	3.8
<i>Sediminibacterium</i>	0.284	6769.1	8404.3	2.91
<i>JGI_0001001_H03</i>	0.299	9699.3	9479	-2.05
<i>Fimbriimonadaceae</i>	0.302	16516	3387.2	-3.82
<i>Candidatus_Moranbacteria</i>	0.302	43705	14587	-4.16
<i>Acinetobacter</i>	0.308	489920	1576500	5.74
<i>Cellvibrio</i>	0.328	9171.8	67315	4.46
<i>Aquabacterium</i>	0.328	145580	46592	-4.69
<i>Ancalomicrobium</i>	0.336	2210.2	178.7	-3.01
<i>Pseudorhodobacter</i>	0.336	64514	11517	-4.42
<i>SWB02</i>	0.354	33613	15503	-3.96
<i>Porphyrobacter</i>	0.354	10386	14009	3.26
<i>Ornithinibacter</i>	0.354	13400	8989.1	-3.34
<i>Pseudarcicella</i>	0.36759	57111	461800	5.31
<i>Duganella</i>	0.36759	6314.2	11566	3.42
<i>Deinococcus</i>	0.36934	6765.1	333.54	-3.51
<i>SM2D12</i>	0.37038	1097.4	2704.6	2.91
<i>Solitalea</i>	0.37038	937.55	3288.5	3.07
<i>Limnohabitans</i>	0.37167	259520	409870	4.88
<i>Fimbrioglobus</i>	0.37838	38004	15341	-4.05
<i>Stenotrophomonas</i>	0.39214	2706.3	1468.5	-2.79
<i>Arcobacter</i>	0.39214	87528	4013.9	-4.62
<i>Zoogloea</i>	0.40055	33968	6352.5	-4.14
<i>Tepidisphaeraceae</i>	0.43216	5706.7	391.6	-3.42
<i>Pedomicrobium</i>	0.43216	1303.9	5831.6	3.36

<i>Candidatus_Kaiserbacteria</i>	0.43216	11284	1954.1	-3.67
<i>Pseudoxanthomonas</i>	0.43362	24871	3552.9	-4.03
<i>Pir4_lineage</i>	0.43362	26211	14179	-3.78
<i>Bosea</i>	0.43362	13521	5096.7	-3.62
<i>Methylophilus</i>	0.43755	5444.7	1959.4	-3.24
<i>hgcl_clade</i>	0.43755	50849	40487	-3.71
<i>Prostheco bacter</i>	0.45276	15884	4887.3	-3.74
<i>Prosthecomicrobium</i>	0.454	10258	2084.4	-3.61
<i>WPS_2</i>	0.454	5373.6	3230.7	-3.03
<i>Bryobacter</i>	0.47066	3324.7	8337.7	3.40
<i>Arenimonas</i>	0.49326	43403	10032	-4.22
<i>Candidatus_Peregrinibacteria</i>	0.50077	30677	783.2	-4.17
<i>Candidatus_Competibacter</i>	0.50077	14123	2741.2	-3.76
<i>Aurantimicrobium</i>	0.50077	27478	125230	4.69
<i>Phreatobacter</i>	0.50077	6394.3	1566.4	-3.38
<i>Mesorhizobium</i>	0.50077	7011.2	3517.5	-3.24
<i>SH_PL14</i>	0.51125	1657.9	2737.3	2.73
<i>Trichococcus</i>	0.51125	2736.1	3816.6	2.73
<i>Luteolibacter</i>	0.51322	51710	40179	-3.76
<i>Acetobacter</i>	0.55039	11763	2584.9	-3.66
<i>Staphylococcus</i>	0.55039	4387.3	162.46	-3.32
<i>Hyphomicrobium</i>	0.55703	11904	31199	3.98
<i>Roseomonas</i>	0.56383	119760	5365.3	-4.76
<i>Fluviicola</i>	0.56891	54551	72915	3.96
<i>Candidatus_Planktoluna</i>	0.57475	11018	91759	4.61
<i>Iamia</i>	0.57475	5096	2643.3	-3.09
<i>Noviherbaspirillum</i>	0.57475	27949	7816.6	-4.00
<i>Ahniella</i>	0.57475	5921.8	3230.7	-3.13
<i>Pseudorhodoplanes</i>	0.59276	3150.2	3585.6	2.34
<i>Cloacibacterium</i>	0.59276	6588.4	7440.4	2.63

<i>Altererythrobacter</i>	0.60501	12230	17266	3.40
<i>Kapabacteriales</i>	0.60635	10491	1188.1	-3.67
<i>Sphingorhabdus</i>	0.60904	104460	47088	-4.46
<i>Pseudomonas</i>	0.61045	250530	322740	4.56
<i>Nitrosomonas</i>	0.63027	4158.6	9024.5	3.39
<i>Candidatus_Accumulibacter</i>	0.63283	26234	7636.2	-3.97
<i>Bacillus</i>	0.63283	5779.2	2001.3	-3.28
<i>Delftia</i>	0.63283	1980.6	471.13	-2.88
<i>Candidatus_Kerfeldbacteria</i>	0.63283	1329.1	489.5	-2.62
<i>Romboutsia</i>	0.63283	27698	5384.5	-4.05
<i>Rhizorhapis</i>	0.63283	2915.3	146.21	-3.14
<i>Thermomonas</i>	0.63499	22652	46784	4.08
<i>Acidovorax</i>	0.64048	54814	32378	-4.05
<i>Arcicella</i>	0.65353	10118	12452	3.07
<i>Rhodoluna</i>	0.65353	72965	278950	5.01
<i>Ilumatobacter</i>	0.65353	7223.9	12309	3.41
<i>Sphaerotilus</i>	0.65353	11321	31017	3.99
<i>SH3_11</i>	0.65353	2972.7	2735.8	-2.08
<i>Aeromonas</i>	0.67561	45251	101720	4.45
<i>Phaeodactylibacter</i>	0.68017	2099.6	10066	3.6
<i>Paludibacter</i>	0.68017	2281.7	1108.5	-2.77
<i>Pseudarcobacter</i>	0.70411	37511	109600	4.56
<i>Clostridium_sensu_stricto_1</i>	0.70812	23070	2006.3	-4.02
<i>Bacteroidetes_vadinHA17</i>	0.70812	2592.1	4887.6	3.06
<i>Tabrizicola</i>	0.72011	3293.9	1667.7	-2.91
<i>Babeliales</i>	0.72011	785.86	583.7	-2.01
<i>OM60(NOR5)_clade</i>	0.72011	839.11	194.95	-2.51
<i>Lacunisphaera</i>	0.72605	3478.6	2572.4	-2.66
<i>LWQ8</i>	0.72605	15771	5018.2	-3.73