THE FACTORS AFFECTING BACTERIAL COLONISATION ON MICROPLASTICS AND THE IMPACT OF TERTIARY TREATMENT OF WASTEWATER ON THE ATTACHED BACTERIA AND MICROPLASTICS

Submitted in fulfilment of the degree of Master of Applied Science: Biotechnology in the Faculty of Applied Sciences at the Durban University of Technology

Saieshna Rajcoomar

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Supervisor : Prof. Faizal Bux

Co-Supervisor : Prof. Sheena Kumari

Co-Supervisor : Dr. Isaac Dennis Amoah

APPROVAL

I hereby approve the final submission of the following thesis.

Prof. Faizal Bux

Supervisor

DTech: Biotechnology

Prof. Sheena Kumari Kuttan Pillai

Co-supervisor

Ph.D.: Biosciences

Dr Isaac Dennis Amoah

Co-supervisor

Ph.D.: Health Sciences

DECLARATION

I hereby declare that this thesis entitled "**The factors affecting bacterial colonisation on microplastics and the impact of tertiary treatment of wastewater on the attached bacteria and microplastics**" submitted for the degree of master's in applied sciences (Biotechnology) at the Durban University of Technology:

1. This is my original work and has not been submitted for a degree at any other university.
2. I further declare that a detailed reference list has been provided on all the sources cited or quoted

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	13/09/2022
Signature of Co-Supervisor	**Date**
	05/12/2022
Signature of Co-Supervisor	**Date**

Dedication

To my parents.

For their love, understanding and support

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Abstract

Microplastics (MPs) in aquatic environments have become an environmental concern globally. In addition to the direct impact of these plastics on aquatic organisms, their surfaces could serve as a unique habitat for various microbial communities through the formation of biofilms. Various factors could play a role in microbial attachment and biofilm formation in wastewater. This study aimed to assess potential factors that lead to biofilm formation on different types of MPs in wastewater and determine the impact of UV and chlorine treatment on these biofilms. In a laboratory scale experiment, MPs (low density polyethylene (LDPE), high density polyethylene (HDPE), and polypropylene (PP) were exposed to untreated wastewater under various conditions of temperature (20°C, 25°C and 35°C), light and dark conditions, as well as aerobic and anaerobic conditions for a period of five weeks. The formation of biofilms on MPs was quantified using optical density (OD_{660}) measurements. The highest biofilm formation was observed in week 3, with an OD of 1.77. Thereafter, a decline in OD was observed, reaching an OD of 1.1 by week 5. This change in biofilm concentration over the week corresponded to changes in nutrient (nitrite, nitrate and ammonia) concentration in the media. A positive correlation was observed between the changes in biofilm concentration and nitrite (r = 0.824) and ammonia (r = 0.1) levels in the media. Meanwhile, a negative correlation observed for nitrate concentration (r=-0.673). Factors such as dark conditions, 25 C, and aerobic conditions presented the highest median biofilm formation with an OD value of 1.6, 1.7 and 1.6, respectively. It was also observed that polyethylene had higher biofilm concentrations compared to the polypropylene. Furthermore, rough MPs had higher biofilm formation than smooth MPs, with median ODs of 1.7 and 1.6 respectively. The microbial communities in the biofilms and wastewater medium were characterised by 16S rRNA amplicon sequencing. The results revealed that the alpha diversity (richness, evenness, and diversity) was lower in wastewater compared to the biofilms. It was observed that PP supported the most diverse bacterial community (H'= 2.51138 and Simpson index= 11.096), while HDPE supported the least diverse bacterial community (H'= 0.88779 and Simpson index= 1.5324). Beta diversity using the Jaccard distance index revealed that the most similar communities were observed among biofilms from the three types of MPs while the most dissimilar communities were observed between the biofilm and wastewater medium communities. The most dominant phyla in both the biofilms and wastewater medium during the five weeks were Proteobacteria, Bacteroidetes and Planctomycetes. The bacterial communities, however, varied for each type of plastic and the wastewater medium. It was observed that *Methylotenera*, *Hydrogenophaga*,

and *Rhodanobacter* was the most abundant genera in biofilms whereas C39(45.25%) and *Luteimonas*(18.96%) were the abundant genera in the wastewater medium. *Methylotenera mobilis* was the most common species among the three types of MPs. In addition, pathogenic species such as *Mycobacterium arupense* and *Methylobacterium adhaesivum* were detected in abundance on LDPE and PP. To assess the impact of UV treatment and chlorination on the attached biofilms, the microplastics with attached biofilm were exposed to UV-C and Chlorine (5 mg/L) treatment for 60 minutes. The biofilms were inactivated (100%) after 30 mins of UV treatment, whereas 10 min was sufficient to achieve 100% inactivation of biofilm by chlorine treatment. In conclusion, the research presented in this study has made substantial contributions to our understanding of the role that environmental factors play in the formation of biofilm on MP surfaces.

Keywords: Microplastics; wastewater; biofilms; 16S rRNA analysis; tertiary treatment

Preface

Research Outputs:

- Journal Article:

  Rajcoomar, S., Amoah, I.D., Abunama, T., Mohlomi, N., Bux, F., and Kumari, S. 2022. Microbial community profile of biofilms associated with microplastics in wastewater under selected conditions and their inactivation. *International Journal of Environmental Science and Technology* (**Under review**)

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Chapter 1: Introduction

1.1 General Introduction

Plastic debris in aquatic environments has emerged as a major global pollution issue (Rummel *et al*. 2017). Plastics are synthetic polymers that are obtained as a result of the polymerisation of monomers extracted from gas or oil (Rasmussen 2018). They are lightweight, durable, cheap, and suitable for manufacturing products used in our everyday life. The durability of plastics makes them highly resistant to degradation, making the discarding of plastics difficult (Pan *et al*. 2020). The increase in the use of plastics has led to an increased discharge of plastics into aquatic environments, including oceans, rivers and lakes. (Carr *et al*. 2016). The larger plastic debris found in aquatic environments are known as 'macroplastics' and they present, environmental issues, economic repercussions, reduce aesthetic value of the aquatic environment and negatively affect aquatic animals (Winton *et al.* 2020). 'Microplastics' has become an increasing environmental concern (Backhaus and Wagner 2020).These emerging contaminants are ubiquitous in the aquatic environment and pose a threat to the biota (Harrison *et al*. 2018).

Microplastics (MPs) are particles that are 5 mm and smaller in size (Shen *et al.* 2019); and are divided into two categories: primary and secondary MPs. Primary MPs are manufactured for industrial purposes, such as personal care and cleaning products, while secondary MPs are formed as a result of fragmentation/degradation of larger plastic material (Talvite *et al*. 2017). They are primarily made up of polyethylene (PE), polypropylene (PP), polyester and other polymers (Carr *et al*. 2016). MPs in aquatic surfaces originate from land-based sources, including urban runoff and aquatic based sources, and form plastic particles through the degradation or breakdown of macroplastics (Birch *et al*. 2020).

Wastewater is recognised as a land-based source of MPs and is known as one of the pathways for MPs to enter aquatic environments. It has been reported that the MPs that have been detected in marine environments resemble the types of MPs found in wastewater effluent (Ziajahromi *et al.* 2016). Moreover, the MPs in wastewater effluents are becoming more dangerous because of the potential for adsorption of harmful agents, including pharmaceuticals and pathogenic organisms, onto their surface (Ziajahromi *et al.* 2017). Nutrients from wastewater can adsorb to these MPs, which form a conditioning layer that further allows the attachment of bacteria and other microorganisms (Khatoon *et al*. 2014). The formation of biofilms takes place by the secretion of extra polymeric substances (EPS) by microorganisms (Rummel *et al*. 2017). The EPS layer contains proteins and glycolipids that provide a structural architecture that forms a matrix around the microorganism, making it possible to attach to various surfaces (Rather *et al.* 2021).

Various factors contribute to the development and composition of biofilms on MPS. These include environmental conditions, MP size, plastic surface properties, and substrate type (Carreres Calabuig *et al*. 2019). At wastewater treatment plants (WWTPs), factors such as the increase in flow velocity, water temperature, nutrient concentration, and other operational parameters such as rate of mixing could play a role in the attachment process. The type and size of MPs also play a role in the attachment of biofilms. Biofilm attachment occurs readily to rough plastic hydrophobics that are small in size (He *et al*. 2021). The formation and existence of biofilms on MPs promote the attachment of pollutants such as persistent organic pollutants (POPs), heavy metals and antibiotics (Jose and Jordao 2020). Additionally, MPs with biofilms could also promote the accumulation of antibiotic-resistant genes (ARGs) and act as a carrier of pathogenic microorganisms (He *et al*. 2021). Due to their hydrophobicity, MPs usually float on the water surface, making it easier for attached pollutants and pathogens to be transported through the environment (Jose and Jordao 2020).

1.2. Rationale of the present study

The occurrence of MPs in aquatic bodies has become an increasing environmental concern globally. Moreover, the surface of MPs could act as a unique habitat capable of carrying various microbial communities through the formation of biofilms. This may facilitate horizontal gene transfer between the attached microorganisms and contribute to the release of pathogens into the environment. WWTPs receiving their influent from industrial and domestic settings could have high levels of MPs and may not be easily removed from wastewater during treatment due to their smaller size. The biofilms also serve as a protective shield against tertiary wastewater treatments aimed at the inactivation of pathogenic microorganisms. Therefore, the focus of this study was to investigate the factors that facilitate bacterial attachment to microplastic particles in wastewater, the microorganisms that are present in the biofilms, and the effect that tertiary wastewater treatment (chlorine and UV treatment) has on both biofilms and attached microorganisms.

1.4. Aim

To investigate the factors affecting biofilm formation to MPs in wastewater and to evaluate the effect of chlorine and UV treatment on MPs and attached biofilms.

1.5. Objectives

i. To determine the effect of selected factors on the attachment of biofilms onto MPs under controlled conditions. *(Chapter 3)*

ii. To investigate the bacterial population dynamics of the biofilms formed under different conditions *(Chapter 4)*

iii. To determine the impact of chlorination and UV treatment on MP-bound microorganisms *(Chapter 4)*

Chapter 2: Literature Review

2.1 Plastic debris in the environment

In the last century, polymer science has been one of the most revolutionary research areas. This research area was influenced by the first discovered synthetic plastic, Bakelite, in 1907 (Frias and Nash 2019). Since then, plastics have become an integral part of our daily lives in homes, offices, grocery shops, schools and even hospitals. Plastics fall in the class of synthetic organic polymers made up of long, chain-like repeating chemical structural units (or 'mers') with a high average molecular weight (Law 2017). Various common plastics composed of hydrocarbons, are usually obtained from fossil fuel feedstocks (Am. Chem. Counc 2015). In the conversion process of resin, many components such as UV and thermal stabilisers, flame retardants, plasticisers, antimicrobial and colouring agents are added. These additives enhance the performance and appearance of plastic products (Law 2017). Plastic material, therefore, possesses highly desirable properties, including durability, strength, low density, low electric and thermal conductivity and resistance to corrosion, giving them water and oxygen barrier capabilities (Frias and Nash 2019) and they can also take on many forms (Figure 3) including foams, fibres and rigid or flexible solids (Law 2017). Plastic debris is distributed in the environment in various forms and size variations (Table 1), such as macroplastic, mesoplastic, microplastic, and nanoplastic (Thushari *et al*. 2020). The low price of plastic materials contributes to their rapid manufacture and widespread usage as food packaging and for medical and technological applications. However, the revolutionary material is slowly becoming a threat to aquatic and terrestrial environments (Frias and Nash 2019).

Table 1: Classification of plastic debris in the environment (Andrady 2017)

Class	Size ranges	Visualization
Macroplastics	>2.5cm	Naked eye
Mesoplastics	0.5cm- 2.5cm	Naked eye and optical microscope
Microplastics	≤5mm	Optical microscope
Nanoplastics	<1µm	Electron microscope

2.2 Microplastics

2.2.1 What are microplastics?

Natural environmental conditions such as solar radiation, abrasion and interaction with organisms cause the slow degradation and fragmentation of larger plastic items resulting in smaller plastic particles which are known as 'microplastics' (Frias and Nash 2019). Microplastics (MPs) are defined as synthetic polymer particles that have a diameter of ≤5mm (Shen *et al*. 2019). They are categorized based on their origin as primary and secondary MPs (Cristaldi *et al*. 2020). MPs that are manufactured for personal care products, cosmetics and detergents are referred to as primary MPs (Blair *et al*. 2017). MPs that originate from the breakdown of larger plastics are referred to as secondary MPs (Talvite *et al*. 2017). They can also be classified based on their shape and polymer type, as illustrated in Figure 2. MPs are typically found as pellets, fibres or fragments in the aquatic environment and are made up of a variety of polymer types including PVC, Polyester, PE, PP and polystyrene (Embrandiri *et al*. 2020). The presence and behaviour of MPs in the environment can be influenced by various material characteristics (Table 3) (Andrady 2017).

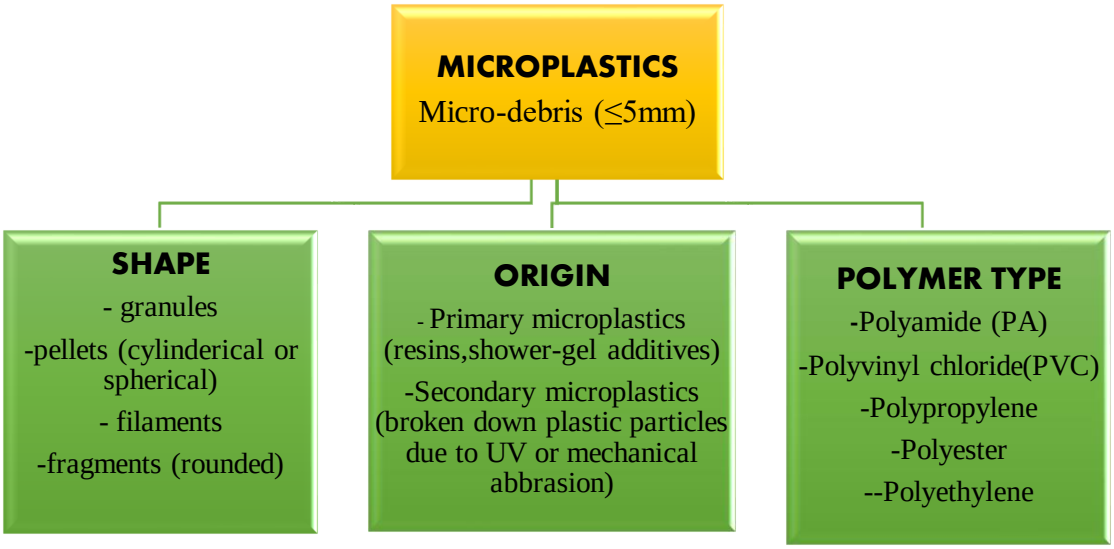

Figure 1: Classification of MPs based on shape, origin and polymer type (Embrandiri *et al*. 2020)

2.2.2 Types of microplastics

There are two categories of polymers: polymers that can be melt processed and polymers that cannot be melt processed. 'Thermoplastics' is the category of polymers that can be recycled, and melt processed to produce useful products. The five major MPs that form part of thermoplastics are: PP, PE, polyvinyl chloride (PVC), polystyrene (PS) and polyethylene terephthalate (PET) (Andrady 2017). The second category of polymer that cannot be melt-processed due to their cross-linked structure is identified as 'thermoset polymers'. Polyurethane foams (used in floats, epoxy adhesives and paints), rubber tyres and reinforced unsaturated polyester composites (GRP) that are used in vessel fabrication are examples of thermoset polymers that contribute to MPs in the environments (Andrady 2017). The different types of MPs can be used in various commercial applications, as seen in Table 2.

The most common MPs found in aquatic environments are reported to be either PE, PP, PVC or PS (Xu *et al.* 2020). There are different grades of polyethylene which include: linear low-density polyethylene (LLDPE), low density polyethylene (LDPE) and high-density polyethylene (HDPE) which are required for various industrial applications (Roex *et al.* 2013). Polyethylene's differ in strength, weight, crystallinity, weatherability and in their chemical structure. LLDPE and LDPE resin are used to produce plastic bags whereas HDPE is moulded to produce milk and chemical jugs. The primary MPs used in PCPs are mainly composed of PE and may contain PP (Roex *et al*. 2013). Secondary MPs are composed of PE, PP, and PS which are often used in packaging and PVC and PES that are commonly used in construction and textiles (Xu *et al.* 2020). MPs consists of different forms (Figure 3): fibres, fragments, films, pellets, beads, and styrofoam (Amelia *et al*. 2021). The forms of MPs typically pollute the environment are fibres or fragments, PE, or PP material that is contributed by anthropogenic, urban, fishery, or marine activities (Amelia *et al.* 2021).

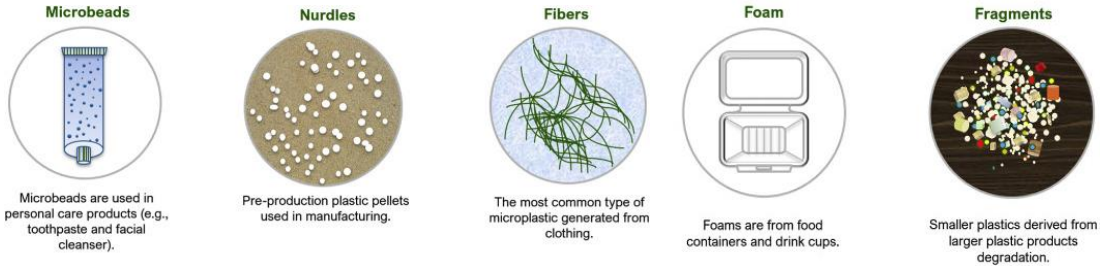

Figure 2 : Common types of microplastics (Wu *et al*. 2019)

Table 2 : Types of MPs and their Commercial uses (Embrandiri *et al*. 2020)

Type of MPs	Commercial Application
Polyamide (PA)/Nylon	Fishing nets and ropes, carpets, sportswear, textiles, film, insulation, roofing materials,
Polyvinyl chloride (PVC)	Piping
Polypropylene (PP), Polyterephthalate (PET), Polyester (PES)	Rope, bottle caps, gear. Strapping, bottles, boats, textiles
Polystyrene (PS)	Cool boxes, floats, cups, utensils, take away packs.
Polyethylene (PE) [HPDE and LPDE	Plastic bags, storage, Containers for milk, cleaning agents, shampoo, boxes, straws,
Cellulose acetate (CA)	Cigarette butts

Table 3: Material characteristics that influence the behavior of MPs (Andrady 2017)

Characteristic	Influence on behavior of MPs
1. Density	Buoyancy determines where in the water column will the MP be present
2. Partial crystallinity	Oxidative degradation and fragmentation due to weathering is determined by the degree of crystallinity
3. Oxidation resistance or weatherability	Chemical structure will determine how easily oxidizable the plastic will be in the environment. Extensive oxidative degradation results in fragmentation

4. Biodegradability	Determines the rate of mineralization and potential partial removal of plastics from the water column or sediment
5. Residual monomer	Toxicity to aquatic organisms that ingest MPs
6. Transport	Bioavailability of residual monomers, additives, and POPs sorbed by the MPs depends on their leaching rates in the gut environment.
7. Additives	Contribute to the adverse impacts on ingesting species
8. Surface Properties	Rate of chemical or biological changes of floating debris determines rate of weathering and sinking of MPs

2.2.3 Sources of microplastics in the aquatic environment

The major sources of MPs in the aquatic environment are classified into two main categories (i) land-based sources, which mostly includes run-off that contains MPs after heavy rainfall and (ii) aquatic-based sources, which are large plastic objects discarded by humans that degrade and form MPs in the aquatic environment (Ziajahromi *et al.* 2016).

I. Land based sources

Runoff containing MPs is from land-based sources, which may be made up of both primary and secondary MPs (Viveknand *et al.* 2021). MP sources include plastic ingredients used in personal care and cosmetic products. These synthetic solid materials are non-degradable and water insoluble and manufactured with polymeric additives that offer the materials the desired functionality (Pereao, Opeolu and Fatoki 2020). PE makes up almost 93 % of the

microbeads used in cosmetics together with PP and other polymers (Gouin *et al.* 2015; UNEP 2015). Wastewater treatment plants (WWTPs) have been reported to be one of the main pathways through which MPs are transported to the aquatic environment (Shcell *et al*. 2021). Once the wastewater from domestic settings enter WWTPs, majority of the microbeads could get captured in sewage sludge; however, due to their small size, a fraction could escape the treatment process and eventually travel to the aquatic environment (Sun *et al*. 2019). Tyre wear and tear particles released through mechanical abrasion also contribute to the MPs flow into the aquatic environment (Kole *et al.* 2017). MPs are also used in a range of medical practices, such as drug delivery systems which may be transported through sewage, and wastewater treatment works into aquatic environments (Masura *et al*. 2015; Smith *et al*. 2018). During the washing process of carpeting and garments, they can also breakdown, releasing microfibres into waterways which eventually enter the aquatic environment (Mathalon and Hill 2014).

II. Aquatic based sources

MPs found in aquatic environments are considered secondary MPs and originate from the degradation of discarded larger plastic objects from anthropogenic activities, such as littering, fishing and shipping activities (Vanapalli *et al*. 2021). Approximately 18 % of the plastic debris found in the ocean environment originate from the fishing industry (Kelly *et al*. 2020). Paint chips from boats and ships and fibres from ropes and nets used for aquaculture contribute to plastics debris in the oceans (Duis and Coors 2016). The build-up of plastic materials along shorelines as a result of discharges from estuaries, port activities and materials used in packaging that breakdown into microscopic fragments are also aquatic-based sources of MPs (Pereao, Opeolu and Fatoki 2020).

2.2.4 Distribution of microplastics in different environment matrices

Due to the extensive industrial and domestic use of MPs, the occurrence of plastic particles as a pollutant in the aquatic environment, including rivers, lakes and marine environments, has recently become an increasing environmental concern (Ziajahromi *et al.* 2016). MPs have been widely detected in aquatic environments and in aquatic organisms throughout the world.

i. Freshwater (rivers and lakes)

The abundance and distribution of MPs in freshwater lakes and rivers result from fishery, agriculture and aquaculture activities. Some components of fishing tools contain nylon particles that were found to contribute to MP pollution in freshwater systems (Yuan *et al.* 2019). Agricultural activities such as plastic mulching used for water retention, soil improvement and many other activities cause farmland soil to become rich in plastic particles and can be transported into freshwater systems **(**Liu *et al*. 2018; Nizzetto *et al*. 2016). Ding *et al.* (2019) reported that in surface water collected from the Wei River, the abundance of MPs ranged from 3.67 to 10.7 items/L. and in sediment samples, the abundance varied from 360 to 1320 items/kg. They also observed that sites with a MP abundance >6.5 items/L were located in densely populated and agricultural planting areas (Ding *et al*. 2019). There is also a large amount of MPs in freshwater due to aquaculture activities. Fishing gear and fish farming activities are the main sources of MPs in aquaculture (Ding *et al.* 2021). A study done by Gopinath *et al.* (2020) reported the mean concentration of MPs of 5.9 particles/ L in water 27 particles/kg in sediment from the Red Hills Lake of Chennai city, India. They also found that the sources of the MPs were most likely due to fishing activities, waste accumulation surrounding the reservoirs, and waste burning near the lake (Gopinath *et al*. 2020). WWTPs also serve as a pathway through which MPs enter freshwater. MPs can be directly released into freshwater via the effluent that is discharged from WWTPs. They can also be released indirectly into freshwater via WWTP sludge that can be used as fertilizer. (Ding *et al*. 2021;

Sun *et al*. 2019). Another major source of MPs in fresh water is terrestrial runoff. Storms, rainfall and consequently flooding can transport plastics into freshwater from land-based sources.

ii. Marine (beaches and oceans)

The increased use of MPs in industrial and household products are released into sewage systems that eventually flow into the oceans (Xiong *et al.* 2019). In recent years, the concentration of MPs in oceans has increased globally (Gallo *et al*. 2018). Pan et al. (2019) reported that the MP concentration in the Northwestern Pacific Ocean ranges from 640-42000 items/km^2 (Pan *et al*. 2019). The number of MPs found in urban areas is generally higher than in rural areas. In a study done by Song *et al.* (2018), in seawater collected off the coast of South Korea the average abundance of MPs in urban areas was 1051 particles/$m^{3,}$ which was higher than in rural areas that had an average abundance of 560 particles/m^3 (Song *et al.* 2018). In marine water systems, the circulation of MPs depends on their polymer density, implying that low density plastic debris presumably recirculates between beach sediments and seawater more often than high density plastic debris (Graca *et al.* 2017). The final destination of MPs found in marine environments is sediments (Xiong *et al*. 2019). Mu *et al*. (2019) reported that the abundance of MPs in the Bering Sea and Chukchi Sea sediments ranged from 0- 68.9 items/kg dw. Water flow rate, sediment depth, and distance from that shoreline play a role in the MP abundance in sediments. Li *et al*. (2019) found that in the Maowi Sea the sediments in the entrance area had an abundance of 1780–2310 items/kg dw which was higher than the sediments of the estuary (940 items/kg dw).

iii. In Biota

There has been an increasing number of reports of ingestion of MPs by fish species found in oceans, seas and freshwater (Andrady 2017). The greater the variety and quantity of smaller sized MPs of certain colors cause an increase in bioavailability of MPs in marine organisms (Ugwu *et al.* 2021). A study done by Li *et al.* (2020) found the percentages of MPs with sizes <1mm and <3mm were 49% and 95% in fish samples, respectively and the most common shape of MPs was found were fibres. Marine organisms can ingest MPs due to their inability to differentiate between MPs and prey or by the consumption of organisms from lower trophic levels, such as plankton that contain MPs (de Sá *et al*. 2015; Bhuyan *et al*. 2021). Bakir *et al.* (2020) confirmed the presence of MPs in the gastrointestinal tract (GTI) of terrestrial crabs, pelagic fish and coral reef fish. MPs were found in 57% of the crabs collected from the South Pacific and MPs were found in 38% GIT of reef fish collected from Vanuatu. MPs were found to be present in a range of food items such as crabs, fish and Yellow Fin Tuna (Bakir *et al*. 2020) and this may cause potential risks food safety and human health. Additives such as plasticizers are detrimental to living organisms and the environment. They affect the hormonal balance, mortality, reproduction, and neurological development of aquatic species (Tullo 2015). These additives prolong the degradation of plastics and also introduce harmful chemicals to biota and eventually humans, who are major consumers of many marine organisms (Andrady 2017).

2.3 Microplastics in Wastewater Treatment plants (WWTPs)

2.3.1 Types of microplastics found in WWTPs

MP polymers that can be found in WWTPs include HDPE,LDPE, PP, polyethylene terephthalate (PET), PES, PVC and PS. The most common MP found in WWTP is PES fibers accounting for up to 89% of wastewater influent and effluent, followed by PE and polyamide, all of which are used in synthetic clothing, facial scrubs and packaging (Shen *et al*. 2019).

Polymers like PP, PS and polyurethane were also observed in wastewater (Shen *et al.* 2019). According to Yang *et al*. (2019), more than 70 % of MPs detected in wastewater were PET, PS and PP. There are a variety of shapes of MPs that have been reported to be found in WWTP, with the major ones being fibers, fragments, microbeads and foams. Domestic WWTPs are a major source of microfibres and microbeads. A study done by Hongprasith et al. (2020) reported that fibers, fragments and sheets (from plastic bags and packaging) make up the biggest fraction of MPs in WWTPs. Fibres found in WWTPs mainly originate from the washing of clothing or from textile handling activities (Hamidian *et al.* 2021). Microbeads originate from cosmetics and personal care products which are small enough to possibly escape filtration during wastewater treatment (Hamidian *et al*. 2021). Dyachenko *et al*. (2016) reported that in China, microbeads contribute <10% of the total MPs in wastewater effluent; however, 306.9 tons are discharged per year. Fragments result from broken down plastic products used daily or PCPs such as toothpaste (Carr *et al*. 2016). According to Shen *et al*. (2019), fragments account for a percentage average of 28.8% (Sun *et al.* 2019). Foams and films originate from the breakdown of plastic bags and other packaging material. The concentration of foams in WWTPs is very low as compared to fragments and microbeads (Bui *et al*. 2020). Murphy *et al*. (2016) reported the percentage of foams (1.3%) in WWTPs was found to be the lowest, whereas fragments (67.3%), fibers (18.5%), films (9.9%), and microbeads (3.0%) made up a larger percentage of wastewater effluent.

2.3.2 The fate of microplastics in WWTPs

Typical WWTPs are not designed or equipped to remove plastic particles (Leslie *et al.* 2017; Talvitie *et al.* 2017). Wastewater is purified through primary, secondary and tertiary treatment processes prior to discharging effluents into the environment. During the primary treatment, most MPs are removed through screening, and sedimentation processes due to their own

settling, flotation, coagulation-flocculation and filtration processes (Carr *et al.* 2016; Murphy *et al*. 2016; Talvitie *et al*. 2017). Low density MPs tend to collect in the grease layer and are later skimmed off (Carr *et al.* 2016). Secondary and tertiary levels of treatment have advanced processes with a higher removal efficiency of the MPs (Carr *et al.* 2016). Secondary treatment removes suspended and dissolved organic material through the action of microorganisms within large aeration tanks (Murphy *et al.* 2016; Talvitie *et al*. 2017). However, tertiary treatment does not affect MP concentration (Talvitie *et al*. 2017). Disinfection processes, polishing or advanced tertiary treatment, such as filtration through sand and/or activated carbon columns, are carried out in some instances before the treated wastewater is released into receiving waterbodies (Prata 2018). MPs are not specifically targeted at any phase of the treatment process and may escape WWTPs via effluents reaching the aquatic environment (Browne *et al*. 2011). High concentrations of MPs have been reported downstream to WWTPs (Magnusson and Noren 2014; McCormick *et al*. 2014). MPs can also accumulate largely in solid wastewater fractions (Murphy *et al*. 2016). Approximately 98% of MPs entering the WWTPs get entrapped in the sludge fraction (Parker *et al.* 2021).

i. Removal of MPs during primary treatment

Sedimentation, flotation, screening and grit removal are the pre-treatment processes commonly applied in WWTPs (Xu *et al.* 2021). This stage of wastewater treatment is designed to screen influents for large debris, capture floatable material (e.g., grease and oil), and settle solids (Mason *et al*. 2016). The high MP concentrations in raw wastewater are reduced following physicochemical lamellar settling (Dris *et al*. 2015). Early skimming and settling were found to be effective in removing buoyant MP particles and other fibrous residues (Carr *et al*. 2016). MPs which have a higher density than wastewater, are removed easily from the wastewater by physical sedimentation (Xu *et al*. 2021). Fibers are reported to be the dominant polymer type

of MPs that remain in primary sedimentation (Carr *et al*. 2016; Ziajahromi *et al*. 2017). Primary wastewater treatment releases more than 20% of influent MPs into the aquatic environment (Hartline *et al*. 2016).

ii. Removal of MPs during secondary treatment

Secondary treatment operations take place in large aeration tanks or ponds, during which microorganisms remove dissolved and suspended organic remaining in wastewater after primary treatment (Raju *et al*. 2018; Iyare *et al*. 2020). During secondary treatment, additional MPs are removed by entrapment in solid flocs, sedimentation in secondary clarifiers or by ingestion by microorganisms (e.g., protozoa) (Iyare *et al*. 2020). The activated sludge process is the most widely used biological method for urban wastewater treatment (Xu *et al.* 2021). Adsorption to sludge particles can effectively remove dissolved and microscopic biodegradable organic matter and suspended solids, including MPs, from sewage during the sludge wasting process (Xu *et al.* 2021). Extracellular polymeric substances (EPS) is an important component of granular sludge that maintains the granule structure, protects against hazardous substances, and removes MPs (Xu *et al*. 2021). EPS coats the MP surface and alters the surface properties of hydrophobic fragments or increases the relative density of particles, allowing them to be removed from wastewater by sedimentation (Schmitt-Jansen 2017). The most common MP types remaining in the effluent after activated sludge treatment are fibers, fragments and films (Conley *et al*. 2019).

iii. Removal during tertiary treatment

Tertiary wastewater treatment is a combination of physical and chemical processes used to remove inorganic and organic pollutants. These processes involve various filtration processes followed by additional disinfecting treatment (Iyare *et al*. 2020). The removal efficiency of MPs depends on the tertiary treatments, including rapid sand filtration, membrane filtration, disc-filtration and coagulation. Membrane filtration has shown optimal removal of MPs with

microbeads and fibres reported to mainly be remaining in the effluent (Ziajahromi *et al*. 2017). Disinfection of wastewater is required to destroy any pathogens or parasites before discharge and reuse (Brandt *et al*. 2017; Scholz 2016). This is usually the last treatment step; therefore, MPs that reach this process have most likely interacted with microbes in the wastewater as they travelled through the WWTP (Enfrin *et al*. 2019). The most common treatment is chlorination which inhibits enzymatic activities resulting in the death of bacteria (Brandt *et al*. 2017). Commercial plastic polymers have the potential to be physically and structurally altered when in contact with sterilization agents (Kelkar *et al*. 2019). Different types of polymers exhibit different degrees of resistance to oxidative degradation by chlorine. Kelkar *et al*. (2019) reported that the chlorine doses used during wastewater treatment are not strong enough cause chemical changes in certain polymers. They found that HDPE and PP plastics were resistant to chlorination, whereas PS was affected by chlorination, possibly due to the absence of plasticizers (additive) and annealing (thermal treatment) of the plastic (Kelkar *et al*. 2019). Ozonation is another disinfection method that can be used to kill chlorine-resistant microbes by disrupting their cell membranes (Ding *et al.* 2019). PE and PS material can however be oxidised by ozone; therefore, MPs could decrease the amount of ozone molecules present to react with bacteria leaving untreated pathogens in wastewater (Brandt *et al*. 2017). Alternatively, UV radiation can be used where UV light is usually applied to disrupt DNA and inactivate pathogenic microorganisms (Carre *et al*. 2018). However, MPs present could shield microorganisms from UV light and protect them from disinfection (Enfrin *et al*. 2019). MPs such as PE, PP, PVC and PS could undergo photo-initiated degradation as a result of UV light exposure during treatment (Hou *et al.* 2021). UV radiation could cause different morphological changes, which could break down and reconstruct surface chemical groups of MPs. This may alter the surface hydrophobicity and adsorption capability of organic pollutants (Lin *et al.* 2020). Lin et al., 2020 reported that the surfaces of PS and PVC were altered after UV

irradiation, PS became rough and generated flakes and PVC developed wrinkles and nodule-like ridges, whereas PE exhibited resistance to UV irradiation.

2.4 Association of microorganisms with microplastics

2.4.1 Biofilm formation on microplastics

Microorganisms are able to form biofilms on both natural and artificial surfaces such as medical equipment, copper and plastic pipes used in water delivery systems (Keswani *et al*. 2016). MPs in aquatic environments interact with co-occurring microorganisms (Kettner et al., 2019; Macreadie et al., 2018) and over a period of time and favorable conditions biofilm formation occurs (Mishra et al., 2021). The attached microbial communities on the MP surface is known as the 'plastisphere' (Amaral-Zettler et al., 2020; Zettler et al., 2013).The physical properties of plastic make its surfaces a unique habitat capable of carrying various microbial communities (Oberbeckmann, Osborn and Duhaime 2016). The main stages of the biofilm formation process (Figure 4) have been well characterised (Wolferen *et al*. 2018). Once the MPs come into contact with the aquatic environment, they are colonized by microbes within seconds. A conditioning layer containing organic and inorganic substances is formed by adsorption (Rummel *et al*. 2017). The conditioning layer attracts bacteria, viruses and other microorganisms to attach to their surfaces, where they are able to obtain more nutrients (Shen *et al*. 2019). MPs provide these biofilm-forming microorganisms with a stable habitat that helps them resist environmental stresses and improve microbial diffusivity (Keswani *et al*. 2016). The bacterial biofilm on MPs possesses a distinct taxonomic composition as compared to its surrounding aquatic environment (Miao *et al.* 2019). MPs can, however, function as vectors of microorganisms. They can enhance the diffusion of microorganisms into the environment and since they have a strong floatability, they are able to support the existence of the surface microorganisms in water for a longer time (Keswani *et al.* 2016).

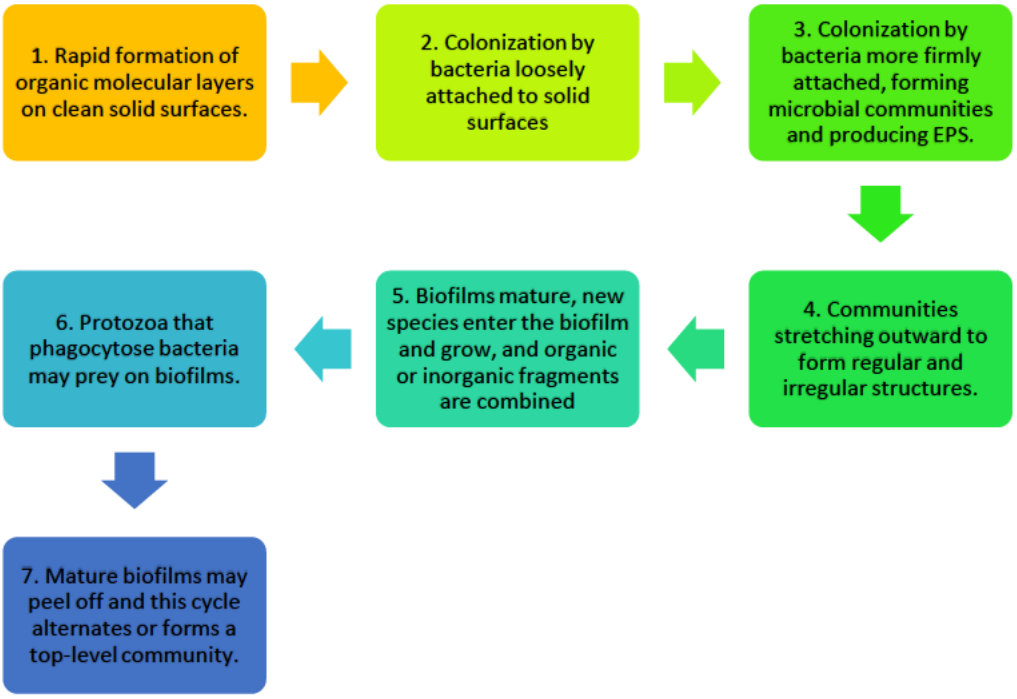

Figure 3: A classic biofilm formation process (Tu *et al*. 2020)

2.4.2 The steps involved in the biofilm formation process

i. Surface attachment

Solid-liquid interface can be responsible for the attachment and growth of microorganisms in biofilms (Jamal *et al*. 2018). Figure 5 shows various steps involved in a biofilm formation process (adapted from Yin *et al*. 2019). The first step in the biofilm formation process is the most important step because it represents the change from planktonic life to biofilm mode (Toyofuku *et al*. 2015). In this step, reversible attachment occurs where bacterial cells attach to surfaces reversibly at the poles of the cells (Carniello *et al*. 2018). Flagella, pili and fimbriae are cell appendages that are involved in reversible attachment. Bacteria can either join the

biofilm life or return to the planktonic life (Toyofuku *et al.* 2015). The next step is irreversible attachment, during which surface proteins (e.g., SadB or LapA) and EPS assist with cell-to-surface adhesion (Petrova *et al.* 2012). Bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) is a second messenger that is involved in the transition from reversible to the irreversible attachment. This messenger is produced by many bacteria and is responsible for the regulation of EPS production and motility in opposing directions (Toyofuku *et al*. 2015). cAMP is another second messenger involved in the transition from reversible to irreversible attachment (Ono *et al*. 2014). The concentration of c-di-GMP and cAMP are controlled by environmental conditions such as carbon and oxygen (McDonough *et al.* 2012). Therefore, the regulation of cell-to-surface attachment by c-di-GMP and cAMP are controlled by the surrounding environmental conditions (Yawata *et al*. 2014)

ii. Biofilm maturation

In this step, the required microbial cell density is achieved through a process known as cell-to-cell communication. This results in the secretion of signaling molecules, known as auto-inducers, that facilitate quorum sensing (Jamal *et al*. 2018). With the assistance of EPS, biofilms form a structured architecture (Toyofuku *et al*. 2015). Bacterial microcolonies develop by assembling previously attached cells and through cell division (Pönisch *et al.* 2018). Growth of these microcolonies occurs by proliferation and begins to produce EPS. EPS makes up more than 90% of the dry mass of mature biofilms. Polysaccharides, nucleic acids, proteins, lipids and other polymers are components of EPS (Flemming 2016). EPS is responsible for cell-to-surface adhesion, the scaffolding of cells and maintaining the architecture of the biofilm (Toyofuku *et al*. 2015). EPS also protects the bacterial cells against stress factors such as antimicrobial agents, host immune systems, oxidation and metallic cations (Flemming 2016).

EPS retain quorum sensing (QS) signalling molecules, extracellular enzymes, and metabolic products within the biofilms (Drescher *et al.* 2014).

iii. Biofilm dispersion/ detachment

This is the final stage where some bacterial cells from the biofilm transfer to planktonic growth and explore other niches and start detaching from the current surfaces (Figure 5). There are two types of dispersal: active dispersal and passive dispersal. Active dispersal depends on cell motility or degradation of EPS, which is triggered by changes in environmental conditions (McDougald et al, 2012). Passive dispersal depends on physical factors such as shearing forces (McDougald et al, 2012). In active dispersal, genes involved in cell motility and EPS degradation are upregulated while genes involved in EPS production and attachment are downregulated (McDougald et al, 2012). A decrease in intracellular c-di-GMP levels that promotes the conversion from the planktonic to the biofilm mode of life will also result in the dispersal of biofilms (Römling et al, 2013). Thus, planktonic cells are dispersed back into the aquatic environment. Detachment/ dispersal of microbial cells and transfer to new niches and surfaces aid in the spread of infections (Jamal *et al.* 2018).

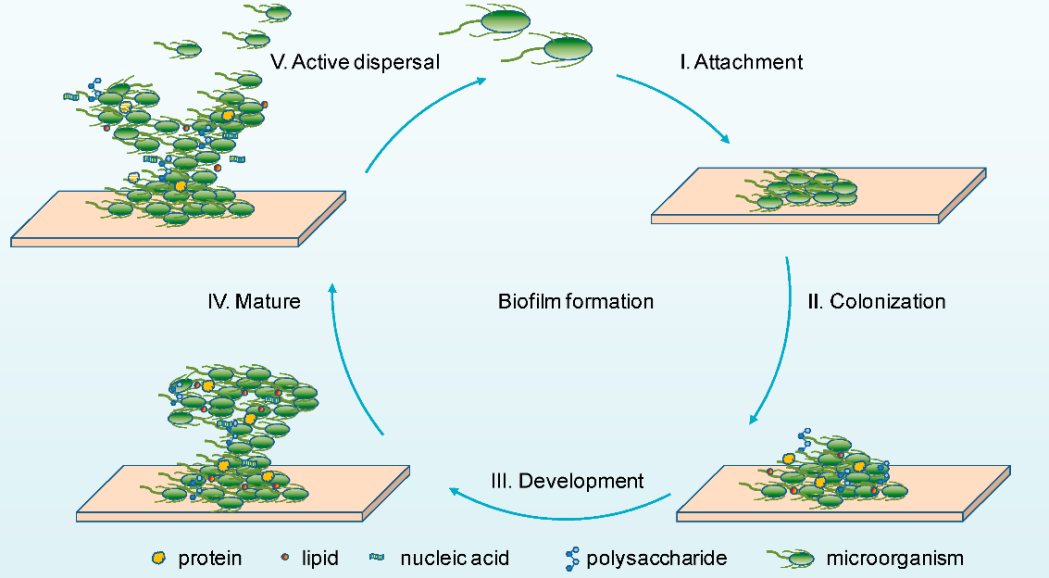

Figure 4: Biofilm life cycle (Yin *et al*. 2019)

2.4.3 The factors affecting biofilm formation

The biofilm formation process occurs over time, and each step is influenced by various physiochemical and environmental factors (Huang *et al*. 2019). The factors that affect the attachment of microorganisms and biofilm formation on plastic surfaces include surface properties of the plastic particles, pH, temperature, oxygen availability, light presence and antimicrobial agents (Khatoon *et al*. 2014). The surrounding environment and cell growth conditions such as carbon source, the composition of nutrient media and growth factors also play a role in the attachment process (Huang *et al*. 2019).

i. Properties of plastic surfaces

Surface charge, surface texture (roughness or smoothness) and surface hydrophobicity are some of the properties of the different types of MP particles found in the environment that may affect the attachment of bacteria to its surfaces (Sooriyakumar *et al*. 2022).

a. Surface Charge

The surface charge determines the binding force between bacteria and the surface, thus affecting biofilm formation. Most bacterial cells are known to be negatively charged; therefore, a surface that is more positively charged is subject to bacterial adhesion and surfaces that are more negatively charged are resistant to bacterial adhesion (Song *et al.* 2015)

b. Surface Energy

Bacterial adhesion can either be enhanced or promoted by altering surface hydrophobicity. The preference for surface hydrophobicity differs among the bacterial species. Previous studies have reported that more hydrophobic surfaces more readily attract bacteria due to the inhibition of hydrogen bridges formed by water molecules (Cai *et al*. 2019).

c. Roughness

Surface roughness enhances the attachment of bacteria to the plastic particle flowing in water (Wang, Guo and Xue 2021). The increase in surface roughness enhances bacterial attachment due to the increase in the contact area between the surface and bacterial cells and protection from shear forces (Al-Amshawee *et al*. 2021; Cowle *et al*. 2019). Biofilm formation can be reduced by smoothening the surface (Mi *et al*. 2018). The effects of surface roughness on bacterial attachment vary with the size and shape of bacterial cells and other environmental factors (Wang, Guo and Xue 2021).

ii. Flow velocity of surrounding aquatic environment

The flow-rate alternates from laminar to turbulent flow, which may vary between locations. Flowing water gives rise to structurally different biofilms depending on the flow rate (Liu *et al*. 2016). During the initial stages of biofilm formation, high flow rates facilitate transport and contact of bulk water microbes onto surfaces as well as nutrient transport (Moreira *et al.* 2013). Increased shear force is known to also boost EPS production, therefore, enhancing cell to

surface attachment (Liu *et al*. 2016) and further providing mechanical stability to growing biofilms (Tallawi *et al*. 2017). High shear stress and turbulent flow promote a thinner, denser, less porous biofilm (Liu *et al.* 2016). However, high flow rates promote detachment of mature biofilms due to the increased shear stress on the outer layer of the biofilm (Moreira *et al*. 2013), which may compromise the microbiological quality of the water. Low flow rates result in weaker, loosely attached, more porous biofilms due to the reduced nutrient transport and shear effects (Tallawi *et al*. 2017).

iii. Nutrient availability

Organic matter in wastewater fuels biofilm formation. Most aquatic microorganisms metabolize biodegradable organic matter for their energy sources and for the production of cellular material (Liu *et al*. 2016). The presence of inorganic nutrients such as phosphorous significantly influences biofilm growth (Pandit *et al*. 2020). Bacteria require phosphorous for growth, cellular metabolism, phospholipid biosynthesis and post-translational control of protein activity (Liu *et al*. 2016). Phosphorus in the form of phosphates is routinely added into metal pipe networks to reduce corrosions (Pandit *et al*. 2020). Phosphate addition also results in changes in the biofilm structure and the microbial community (Pandit *et al.* 2020). Nitrogen is another inorganic nutrient that affects biofilm development. Nitrogen is a building block for proteins and genetic material (Liu *et al.* 2016). Autotrophic nitrifying bacteria are a major biofilm forming microorganisms that use nitrogen -based compounds (ammonia, nitrate, nitrite and urea) as an energy source (Liu *et al*. 2016). Ammonia is often found in untreated wastewater and is a preferred compound for biomass production (Liu *et al*. 2016). Biofilms that are mainly made up of autotrophic bacteria tend to form at high nitrogen-to-carbon ratios, whereas heterotrophic bacteria tend to form at low nitrogen-to-carbon ratios (Liu *et al*. 2016). Trace metals such as iron and copper are known also to affect biofilm growth. Iron is required

for almost all bacterial growth and development, but high concentrations can be toxic to the cells (Liu *et al*. 2016).

iv. pH

The pH of water during the treatment process is often adjusted to promote optimum water treatment, reduce the decay of disinfectants and control corrosion (Ratnaweera *et al*. 2020). Common maintenance of wastewater treatment network is pH at 7 or 8.4 to minimise disinfectant decay (Liu *et al.* 2016). Optimum pHs vary amongst different bacteria (AWWA. 2013). The pH of wastewater could affect cell-to-surface interactions, subsequently their initial attachment onto surfaces (Liu *et al*. 2016). Many biofilm forming microorganisms will have a negative surface charge at ~pH 7 due to the presence of anionic groups (e.g., phosphate) on the cell surface (Liu *et al*. 2016; Palmer *et al.* 2007), resulting in electrostatic repulsion when interacting with negatively charged surfaces. A drop in pH close to the isoelectric pH, due to growth of nitrifiers, may reduce the bacteria-to-surface electrostatic repulsion thus creating a higher potential for bacterial attachment onto surfaces (AWWA, 2013). Biofilm could also form at pH below 7, due to decay of disinfectants and potential changes in surfaces net charge characteristics, making them more prone to bacterial attachment (Liu *et al*. 2016).

v. Water temperature

In multi-seasonal countries, wastewater is often subjected to temperature fluctuations. These temperature fluctuations can significantly affect the initial cell-to-surface attachment and subsequent biofilm formation (Liu *et al*. 2016). Gene expression is affected by temperature fluctuations resulting in changes in the microbial ability to generate EPS and alterations of the cell surface hydrophobicity (Lebre *et al.* 2017). Temperature variations are reported to modulate cell surface in a growth-phase-dependent manner (Liu *et al*. 2016), subsequently affecting their affinity for attachment to the substratum. Biofilm formation at lower

temperatures may vary amongst different microorganisms. Biofilm-forming microorganisms tend to form fewer biofilms at lower temperatures (Liu *et al*. 2016), but some microorganisms form more developed biofilms at these low temperatures (Townsley and Yildiz 2015). The metabolism of bacteria is closely associated with the presence of enzymes (Achinas *et al*. 2019). Biofilm formation depends on the enzyme reaction rate, which is directly affected by temperature. Studies have also shown that bacteria have an increased surface area at low temperatures than at higher temperatures (Govaert *et al.* 2018). The number of flagella from bacteria is temperature dependent. When the number of flagella increases, the surface area of bacteria increases and the chance of bacterial adhesion (Townsley and Yildiz 2015).

vi. Oxygen availability

Dissolved oxygen (DO) concentration is an important factor in the biological wastewater treatment process. DO concentrations affect microbial characteristics such as flocculation and settling properties as well as the removal rate of organic matter (Liu *et al*. 2016). During the transport of MPs through the environment, the attached biofilm forming organism must survive various stressful environmental conditions, particularly high oxygen levels (Liu *et al.* 2016). Low oxygen concentrations are known to induce biofilm, whereas normal oxygen concentrations reduce biofilm formation (Totani *et al*. 2017). Anaerobic and aerobic conditions are known to affect the morphological changes of biofilms which may alter the diffusibility of substances and enable metabolic adaptation by maximizing the exchange of nutrients and waste products (Toyofuku *et al*. 2016).

vii. Effect of light

Light is regarded as an important variable that directly drives the composition of a microbial community (Piwosz *et al.* 2020). The overall biofilm system is shaped by the complex interactions between autotrophic and heterotrophic microbes (Schmidt *et al*. 2018). There are major differences between the biofilm community composition under ambient light conditions

and dim light conditions in dark conditions (Pinto *et al*. 2019). A high abundance of autotrophs can be found under ambient light conditions as light is the primary source of energy for autotrophic microorganisms (Schmidt *et al*. 2018) and a low abundance under dim light or dark conditions (Pinto *et al*. 2019). Light also affects bacterial metabolism. In the presence of light, bacteria take in more organic carbon, including sugars, metabolise them faster. In the dark, those functions are reduced, and the bacteria increase protein production and repair, making and fixing the machinery needed to grow and divide (Pinto *et al*. 2019).

2.5 Microbial diversity of biofilms on different types of microplastics

Biofilms are made up of individual microbial species or a mixed consortia that can also include pathogenic species. The coordination between these communities plays an important role in the exchange of substrate, distribution of metabolic products and the excretion of metabolic end-products (Jamal *et al*. 2018). Biofilm formation is controlled by various genetic pathways, the expression of type IV pili and the production of EPS (Kelly *et al*. 2021). The composition of these plastic associated microbial communities is affected by spatial and temporal variations in environmental parameters.

Previous studies on biofilm formation on plastic surfaces have reported biofilm microbial communities with typical biofilm primary colonizers of the Proteobacteria phylum, such as the genera *Alteromonas* and *Roseobacte*r, followed by members of the phyla Bacteroidetes, Acidobacteria, Actinobacteria, Cyanobacteria, Firmicutes, Planctomycetes and Verrucomicrobia (Pinto *et al*. 2019). A study done by Ashar *et al* (2020) reported Proteobacteria as the most dominant bacterial phylum which made up more than 70% of the microbial population of the biofilm on PET, PS and PE MPs followed by Actinobacteria, Firmicutes, Deinococcus-Thermus, and Bacteriodetes. Genera *Nitratireductor*, *Pseudomonas* and *Cupriavidis* were found to be the most dominant in biofilm communities. Similarly, Wang

et al (2021) found that Proteobacteria, Actinobacteria, Bacteroidetes, Acidobacteria and Cyanobacteria communities were dominant in biofilms attached to PP and PE MPs. Betaproteobacteria and Alphaprotobacteria were the dominant classes found in the biofilms. Vaksmaa *et al* (2021) reported that irrespective of polymer type that the majority of the DNA analysed from biofilms on PE, PP and PS MPs consisted of sequences belonging to the domain bacteria and the most abundant phyla were Proteobacteria, Bacteroidetes, Cyanobacteria, Planctomycetes, Actinobacteria, and Acidobacteria. Several studies found genera *Rhodanobacter, Pseudomonas, Vibrio, Hydrogenophaga* and *Flavobacterium* in plastic biofilms in aquatic environments (Roager and Sonnenschein 2019; Jiang et al. 2018; Ogonowski et al. 2018; Dussud et al. 2018). A study conducted by Zhao et al. (2021) reported the genera found in sewage and biofilms on PVC MPs were *Mycobacterium, Emiticicia, Gemmatimonas, Aquabacterium, Turicibacter* and *Rhodococcus.* The abundance of *Sphingopyxis* were found to be the highest among all genera. *Sphingopyxis* was reported to have the ability to degrade hydrocarbons and is important taxa associated with MPs (Oberbeckmann and Labrenz 2020). *Reyranella, Nitrosomonas, Aquaspirillum* were also highly abundant in sewage and biofilms (Zhao *et al*. 2021). Similarly, Kelly *et al*. (2021) reported genera *Klebsiella, Sphingomonas* and *Pseudomonas* were more abundant on effluent MPs than on sewage MPs. In contrast, genera *Aeromonas* and *Arcobacter* were found to be less abundant on effluent MPs. *Acinetobacter, Arcobacter* and *Aeromona*s were found to be less abundant on sludge MPs compared to sewage MPs (Kelly *et al.* 2021). This could suggest that different bacterial genera colonise MPs and their abundances vary at different stages of their journey through sewage and wastewater treatment.

Wastewater contains many different types of potentially pathogenic bacteria that could colonize MPs during their transport through sewers and wastewater treatment process. MPs have the potential to select for and enrich both pathogenic prokaryotic and eukaryotic microbes

(Kettner et al. 2017, 2019). Zhao *et al*. (2021) reported potential pathogens Mycobacterium and *Legionella* in high abundance in sewage and biofilms indicating that PVC MPs can also adsorb pathogenic bacteria from the surrounding sewage. Kelly *et al.* (2021) reported several potentially pathogenic bacteria associated with human infections. For example, *Acinetobacter* that are a common cause of nosocomial infections. *Klebsiella pneumoniae* and *Klebsiella aerogenes* were more abundant on effluent microplastics compared to sewage microplastic, indicating that these species increased in abundance during the wastewater treatment process (Kelly *et al*. 2021) and could be discharged into the receiving aquatic environments. Both of these *Klebsiella* species are part of the normal human microbiome, but they can also cause opportunistic and nosocomial infections.

Chapter 3: the factors affecting biofilm formation on microplastic particles in wastewater

3.1 Introduction

The aquatic environment is becoming increasingly polluted with plastic debris. The increase in the production and use of plastic materials contributes to the increase in its pollution (Okshevsky *et al*. 2020). Macroplastics are the larger pieces of plastic found in aquatic environments while the MPs are defined as plastic particles that are smaller than 5 millimetres in diameter and are formed by the breakdown of macroplastics (Gong *et al*. 2019; Tu *et al*. 2020). Among the many hazards posed by these pollutants to aquatic ecosystems are the risk of direct ingestion by aquatic animals, transfer and bioaccumulation within the food web, and transmission of harmful heavy metals, organic pollutants, and pathogenic microorganisms (Chapron *et al*. 2018).

When MPs come in contact with water, they are rapidly colonized by microorganisms, forming biofilms (Kelly *et al.* 2021). "Plastisphere" refers to the unique community of microbes that forms on the surface of MPs (Amaral-Zettler *et al*. 2020). It has been recently discovered that microorganisms attached to MPs can be transported and dispersed over considerable distances (Bowley *et al*. 2021). Additionally, it has been known that the MP surfaces with biofilm could potentially promote horizontal gene transfer (HGT) between bacteria, which could lead to transfer of antibiotic-resistant and pathogenic genes between bacterial communities (Karkman *et al*. 2017). This is a major concern as it could facilitate the spread of antibiotic-resistant genes and pathogenic bacteria in the aquatic environment (Shen *et al*. 2019).

WWTPs are one of the major routes for MP particles to enter the environment, and they also provide an ideal environment for the formation of biofilms. MPs are exposed to various physical and chemical factors in wastewater that may influence the development of biofilms

(Tallawi *et al*. 2017; Sajjad *et al*. 2020; Li *et al*. 2022). In general, the development of a biofilm is a complex three-phase process that includes attachment, maturation, and detachment (Saxena *et al*. 2019). Temperature, light/dark conditions, oxygen and nutrient availability are all factors that could affect the biofilm formation process (Akog lu 2020). The hydrophobicity and texture of attaching surfaces are also known to contribute to biofilm formation (Shen *et al*. 2019; Okshevsky *et al*. 2020). For instance, there may be a greater tendency for microorganisms to colonize particles with textured/rough surfaces as compared to particles with smooth surfaces (Shen *et al.* 2019).

As a result of the formation of biofilms, these microorganisms may be protected from surface detachment, predation, inhibitory and degradative mechanisms, as well as from disinfecting processes during wastewater treatment (Rittman 2018). This may pose a significant environmental concern (Sun *et al.* 2019; Liu *et al.* 2019; Rittman 2018) since it may result in the discharge of MPs and microorganisms bound to MPs into the freshwater environment. Although the above information is generally acknowledged, the specific factors involved in the formation of biofilms in wastewater settings remain poorly understood. Therefore, the purpose of this study is to identify those factors or conditions that are likely to facilitate biofilm formation and microbial attachment to microplastics in wastewater, as well as evaluate the effects of tertiary wastewater treatment (chlorine and ultraviolet treatment) on biofilm inactivation using laboratory scale experiments.

3.2. Methodology

3.2.1 Microplastic particles

Three different types of MPs; High-density polyethylene, Low-density polyethylene and polypropylene of varying sizes was purchased from a commercial supplier (Merck (PTY) LTD, South Africa) and were used in this study.

3.2.2 Biofilm formation

In this experiment, exposure time, temperature, light/dark conditions, and aeration/non-aeration were used to determine the conditions affecting biofilm formation on selected types of MPs. The study used untreated wastewater collected from a domestic wastewater treatment plant in Durban as a medium for the experiment. The wastewater was then filtered using a mesh sieve (100 µm pore size). Two grams of MP particles of different types were added to each Erlenmayer flask filled with 100 mL of the filtered wastewater. Following that, the flasks were subjected to different combinations of conditions as detailed in Table 4. The exposure time was tested over a period of five weeks for each experiment to determine the optimum period for biofilm formation on the MPs. In order to understand the effects of temperature, each of the experiments (Experiment 1 to 8) (Table 4) were conducted separately at three incubation temperatures (20°C, 25°C and 35°C).

Table 4: The conditions used for the separate experimental setups at each temperature (20°C, 25°C and 35°C)

Experiments	**Combinations of conditions**	
1	D-A-S	Smooth MPs in dark and aerobic conditions
2	D-A-R	Rough MPs in dark and aerobic conditions
3	D-AN-S	Smooth MPs in dark and anaerobic conditions
4	D-AN-R	Rough MPs in dark and anaerobic conditions
5	L-A-S	Smooth MPs in light and aerobic conditions
6	L-A-R	Rough MPs in light and aerobic conditions
7	L-AN-S	Smooth MPs in light and anaerobic conditions
8	L-AN-R	Rough MPs in light and anaerobic conditions

The dark conditions were achieved by covering the flasks with foil and preventing light from entering, keeping all other parameters similar to those of the light conditions. Aerobic conditions were achieved by covering the flasks with perforated gauze. Anaerobic conditions were generated in sealed reactors that were initially purged with nitrogen gas. All MP types, including rough and smooth MPs, used in this study were exposed to all the conditions described above.

3.2.3 Analysis of Biofilm formation

Optical density (OD) measurements were used to assess biofilm formation on MP particles. This was achieved by collecting MP (10) particles at weekly intervals, washing them with sterile water, and placing them on nutrient broth. Afterwards, the MP particles in the nutrient broth were vigorously shaken both manually and using a vortex for 1 minute to remove the attached biofilms. Following 24 hours of incubation, the OD was measured at 660 nm using a Spectroquant pharo 300 Spectrophotometer.

3.2.4 Nutrient analysis

Wastewater samples were collected from the flasks (used above in 2.2) during week one, week three and week five. The samples were filtered through 0.45 μm syringe filters before the analysis. Nutrient (nitrate (NO_3), nitrite (NO_2), and ammonia (NH_4)) concentration in the wastewater were measured using the Thermo Gallery photometric analyser (Thermo Scientific, UK).

3.2.5 Scanning Electron Microscopy

A scanning electron microscope (SEM) analysis was conducted to confirm the attachment of bacteria to MP surfaces. For this experiment, MPs were fixed in 2.5% glutaraldehyde for 8 hours at 4 °C. After fixation, samples were rinsed with 0.1 M sodium phosphate buffer (pH 7.2) and dehydrated with alcohol series consisting of 30%, 50%, 70%, 90%, 95% and 100%

alcohol (10 min each). In order to minimize distortion of the microplastics prior to SEM analysis, the critical point drying (CPD) was determined. The particles were then mounted onto a stub, covered in carbon glue, and coated with gold palladium. The stub was then placed in the scanning electron microscope and images were recorded and stored.

3.2.6 Statistical analysis

The data was captured in Excel (Microsoft Corp., USA) and diamond plots were used to represent the distribution of the data obtained for biofilm concentrations under the various experimental conditions. Each box plot illustrates the estimated median (centre line), upper and lower quartiles (box limits), interquartile range (whiskers), and outliers (points). The median biofilm concentration values were compared to determine the concentration of biofilms formed on HDPE, LDPE and PP. To determine the significance between light and dark conditions; aerobic and anaerobic conditions; rough and smooth microplastics, the t-test was applied. The two-way analysis of variance (ANOVA) was used to demonstrate the significance of temperature (20°C, 25°C, and 35°C) and MP types (HDPE, LDPE, and PP) on biofilm formation and Pearson correlation coefficient (r) was used to determine the relationship between biofilm concentration and nutrient concentration in the media.

3.3 Results

3.3.1 Relationship between biofilm formation, exposure time and nutrient concentrations in the medium

The formation of biofilm was evident in all experiments regardless of the conditions. As determined by OD value, the highest amount of biofilm formation occurred at week 3 (OD = 1.77), followed by a decline in OD value reaching its lowest point at week five (OD = 1.1) indicating a decrease or detachment of biofilm after a certain period. Additionally, as demonstrated by SEM images shown in Figure A1 and A2, there was evidence of a biofilm formation on these microplastics (Supplementary material).

A comparative analysis of the nutrient concentrations in the wastewater revealed a decrease in ammonia (NH_4) concentrations from 33,45 mg/L to 7,95 mg/L from week 1 to week 5 while nitrate (NO_3) concentrations increased from 0 mg/l to 40,65 mg/l. The concentration of nitrite (NO_2) increased from 0.01 mg/L in week 1 to 0.74 mg/L in week 3, and then decreased to 0 mg/L in week 5 (Figure 5). The correlation analysis revealed a positive linear relationship between biofilm concentration and NO_2 (r = 0.824) and NH_4 (r = 0.1). In contrast, the correlation analysis for biofilm concentration and NO_3 revealed a negative linear relationship (r = -0.673).

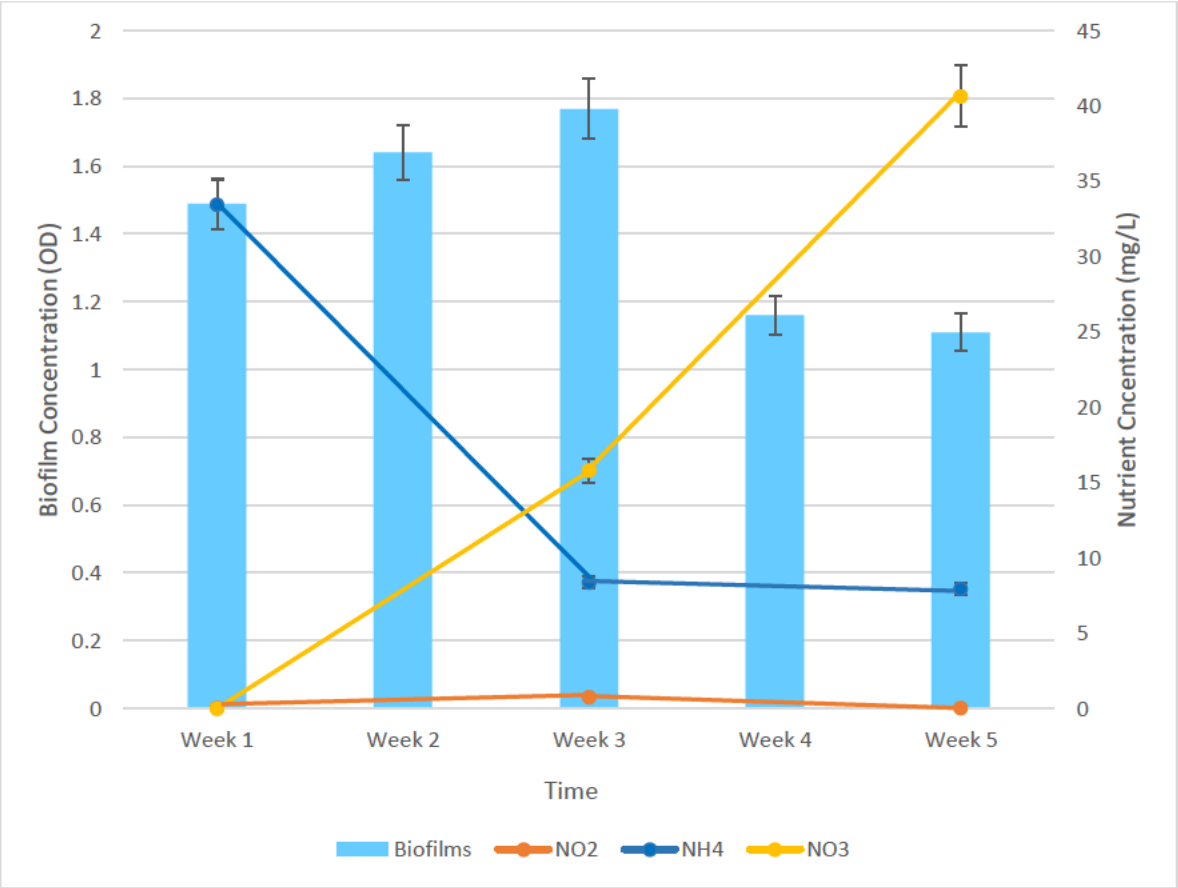

Figure 5: The amount of biofilm formed on microplastic particles and nutrient usage during a period of 5 weeks

3.3.2 Biofilm formation on the different types of microplastics

Median biofilm values on HDPE and PP have remained almost in a similar range from week 1 to week 5. In contrast, the median biofilm values for LDPE varied slightly in each week, as compared with HDPE and PP. According to Figure 6 (A), the highest median biofilm concentrations were observed for LDPE and HDPE on week 3 and slightly lower concentrations for PP (median of 1.75). Likewise, the biofilm concentrations were observed to differ for the three temperatures (20, 25 and 35 °C). The highest median biofilm concentration of 1.65 was observed for HDPE at 20°C. In contrast, at 25°C and 35°C, the greatest median concentration of biofilms was observed for the LDPE MPs, with values of 1.7 and 1.65, respectively. Statistically, the concentration of biofilms on the different types of MPs at various

temperatures did not differ significantly (p-value $\geq$ 0.05). These results demonstrate that the temperature range used in this study did not significantly affect the formation of biofilms on MPs. A similar conclusion was reached when assessing the biofilm concentrations on MPs subjected to light and darkness. In Figure 6 (C), for instance, the highest median biofilm concentrations of 1.7 and 1.6 were recorded on the LDPE MPs exposed to light and dark conditions, respectively. Figure 6 (D) and Figure 6 (E) demonstrate similar results for aerobic and anaerobic conditions, as well as for smooth and rough MPs. In this study, it was discovered that polyethylene MPs (HDPE and LDPE) yielded a higher concentration of biofilms than polypropylene MPs (PP) under the various conditions investigated.

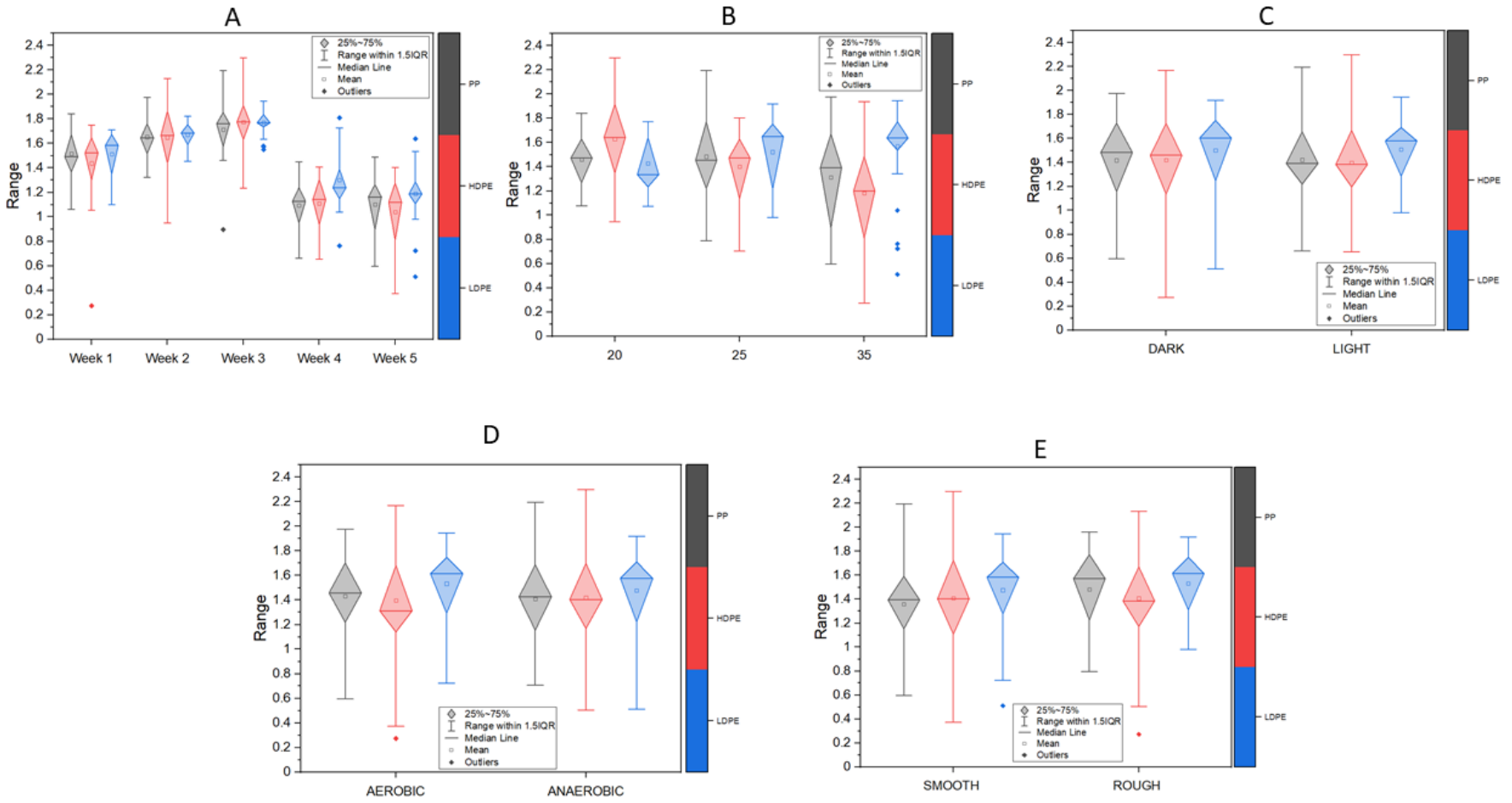

Figure 6 :Diamond box plots showing the difference in biofilm formation on the different microplastics at the different experimental conditions. (A) Impact of duration of exposure on biofilm formation (B) Impact of temperature on biofilm formation (C) Impact of dark or light conditions on biofilm formation (D) Impact of aerobic and anaerobic conditions on biofilm formation (E) The difference in biofilm formation on smooth and rough microplastic particles.

3.3.3 Impact of combined factors on biofilm formation

i. Impact of combined conditions on biofilm formation on PP

The combined conditions that led to the highest impact on biofilm formation differed as the weeks progressed. During the first week, the D-A-R showed the highest median biofilm concentration of 1.8, while the L-A-S demonstrated the lowest. Likewise, the highest median concentration, of 1.2, was observed on the D-AN-S experimental setup at week four, whereas by week five, the highest concentration was observed on the L-A-R experimental setup (Figure 7(A)).

Biofilm formation under the three different temperature conditions varied significantly compared to the concentrations over time. At 20°C, the L-A-R had the highest median concentration (1.7). Whereas at 25°C and 35°C, the highest median concentrations were observed in the D-A-R conditions (Figure 7(B)). Despite these differences, the results further indicated that rough MPs under aerobic conditions facilitated the formation of higher biofilms. The overall highest occurrence of biofilms in this study was observed in the D-A-R combination at 35°C.

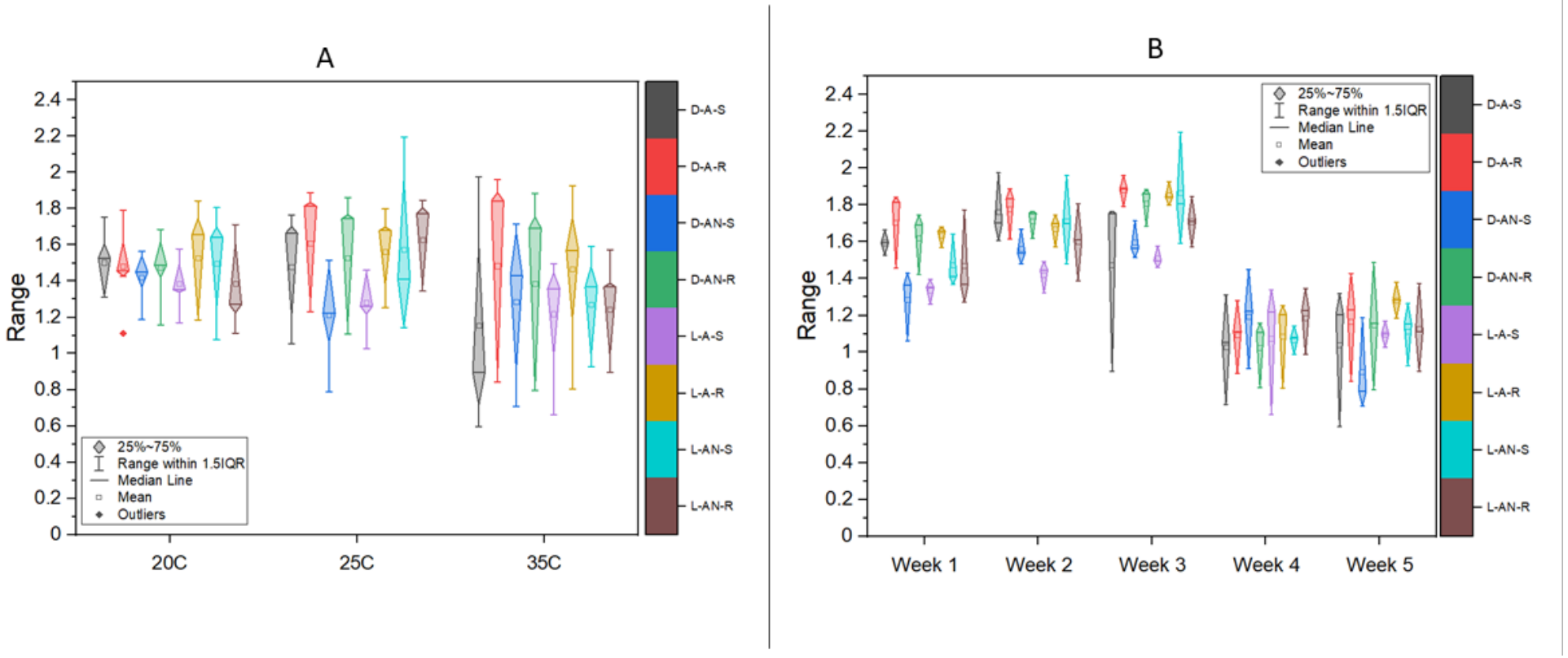

Figure 7: Diamond box plots showing the difference in biofilm formation on PP microplastics for each combination of experimental conditions. (A) Impact of duration of exposure on biofilm formation (B) Impact of temperature on biofilm formation

ii. Impact of combined conditions on biofilm formation on HDPE

As for HDPE, in the first week of study, the D-A-S had a median biofilm concentration of 1.6, while the lowest biofilm concentration was observed in the D-A-R. At weeks four and five, the highest median concentrations (1.2 and 1.1) were recorded on the L-A-R.

The biofilm concentration on HDPE also differed under the three different temperatures. At 20°C, the highest median concentration was found on the D-AN-S. On the other hand, at 25°C, the highest median concentration (1.8) was observed for L-A-R while at 35°C, the highest concentration was observed on the D-AN-R experimental setup (Figure 8(B)). Overall, the highest median biofilm concentration was observed in L-A-R at 25°C.

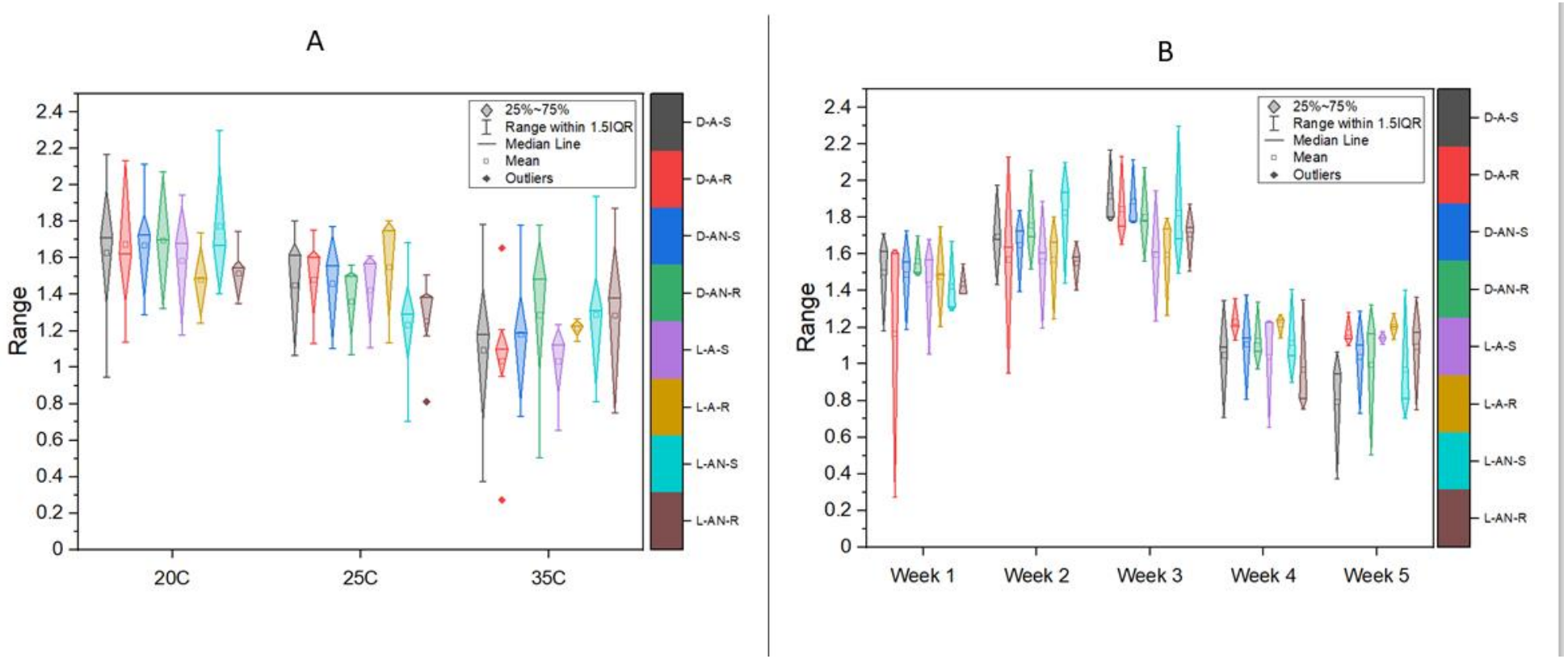

Figure 8: Diamond box plots showing the difference in biofilm formation on HDPE microplastics for each combination of experimental conditions. (A) Impact of duration of exposure on biofilm formation (B) Impact of temperature on biofilm formation

iii. Impact of combined conditions on biofilm formation on LDPE

In this experimental setup, during week one, the D-A-R produced the highest median biofilm concentration (1.7), while the L-AN-R generated the lowest median biofilm concentration (1.3). In general, the combination of conditions that had the greatest impact on biofilm formation varied over time. Figure 9 (A) shows a similar pattern with PP (Figure 7(A)) and HDPE (Figure 8(A)) as well as a decrease in biofilm concentration after week three. By week four the highest median concentration (1.4) was observed on the D-A-R and by week five the highest median concentration (1.3) was detected on the L-AN-S experimental setup. The biofilm concentrations at the three individual temperature conditions varied, but the combined conditions with the highest impact on biofilm formation were similar at both temperatures. Specifically, at 20 °C and 35 °C, the highest median biofilm concentrations of 1.6 and 1.8 were observed for D-AN-R (Figure 9 (B)). At 25°C, however, the highest median biofilm concentration of 1.8 was found on the L-A-R. Despite the differences, results indicated that D-AN-R at 35°C assisted the most biofilm formation.

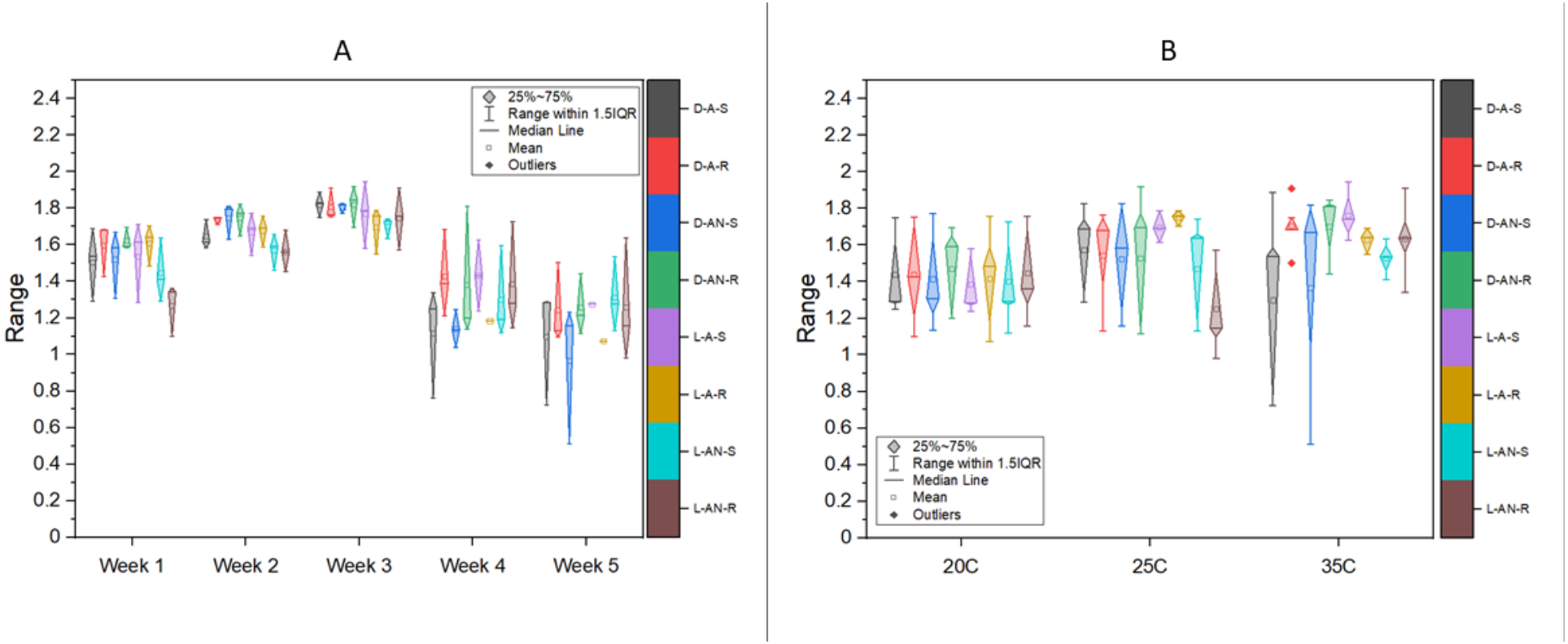

Figure 9: Diamond box plots showing the difference in biofilm formation on LDPE microplastics for each combination of experimental conditions. (A) Impact of duration of exposure on biofilm formation (B) Impact of temperature on biofilm formation

3.4. Discussion

In the process of biofilm formation, the exposure time is crucial. Increasing the duration of exposure to wastewater in this study resulted in an increase in the amount of biofilm attached to the MPs. Figure 5 depicts the development of biofilms on both rough and smooth MPs over a five-week period. The onset of biofilm was observed between weeks one and two, which involves the adsorption of macromolecules (i.e., proteins and polysaccharides) to the surfaces of the plastic particles. This creates favorable conditions for bacterial colonization of the plastic particles (Huang *et al*. 2019). Images obtained by SEM (Figures A1 and A2) show that bacterial cells have begun attaching to the surface of the MP during this period. This stage usually involves both nonselective adhesion and selective adhesion (Huang *et al*. 2019). The second stage of biofilm formation is the process where the biofilm develops and matures. A significant increase in biofilm concentration was observed from week two to week three (Figure 5), indicating increased bacterial attachment and biofilm maturity. Additionally, the SEM images in figure (A1) demonstrate the accumulation of bacterial cells and the formation of a biofilm during this period. As the biofilm matures, it becomes thicker until the nutrients are depleted, which ultimately results in a decrease in biofilm activity. This was apparent in this study, where there was a decrease in biofilm formation between week three and week five, based on a decrease in OD (from 1,77 in week 3 to 1,1 in week 5). This trend was evident in almost all the experimental setups for the different types of MPs. This decrease in biofilm activity is termed the 'aging biofilm state' (Huang *et al*. 2019). During this stage known as the dispersal stage the cell leaves the biofilm (Toyofuku *et al*. 2015).

Biofilm formation is a highly regulated process that occurs over time, as demonstrated in this study, and each step is influenced by various environmental factors (Huang *et al.* 2019). One important factor is nutrient availability. Generally, biofilms are more prevalent in nutrient-rich

environments and are influenced by inorganic nutrients, such as nitrogen, as well as organic matter (Liu *et al.* 2016). These nutrient rich environments encourage the transition of bacteria from a planktonic state to a biofilm form, whereas reductions in nutrient levels result in the detachment of biofilms from surfaces (Sehar and Naz 2016). Carbon, nitrogen, and phosphorus are nutrients necessary for the formation of biofilms (He *et al*. 2021). In wastewater, both heterotrophic and autotrophic nitrifying bacteria can form biofilms. Biofilms have been reported to contain nitrifying bacteria such as *Bacillus* (Liu *et al*. 2016). In addition to serving as a key nutrient in biofilm formation, nitrogen also serves as a building block for proteins and other genetic materials (DNA and RNA). In this study, a positive correlation was found ($r=0.677$) between the concentration of biofilms and the concentration of NO_2 and NH_4 in the media under aerobic conditions. In contrast, the concentration of biofilms exhibited a negative correlation with NO_3 under similar conditions. It may be that the decline in biofilm formation in week 5 was due to a decrease in nutrient concentration, which resulted in ageing of the biofilm and subsequent detachment from the microplastic. Furthermore, it was noteworthy that under aerobic conditions, the conversion of ammonia to nitrite and nitrate was observed at various stages of biofilm development, suggesting the presence of nitrifying bacteria as a dominant microbial community within the biofilm. The accumulation of nitrate under aerobic conditions suggests that denitrifiers are not active in these conditions. This suggests that the composition of wastewater and operational/environmental conditions may influence the structure of microbial communities within biofilms (Liu *et al.* 2016).

Biofilm formation may be influenced by temperature as the enzyme reaction rate is directly affected by temperature. Additionally, studies have shown that bacteria have a greater surface area at low temperatures than at higher temperatures (Govaert *et al*. 2018). In a study conducted by Townsley and Yildiz (2015), biofilms formed by *Vibrio cholerae* at 15 °C and 25°C demonstrated greater thickness and better structure than biofilms formed at 37 °C. Studies have

also linked the temperature effect with the number of bacterial appendages. For example, when the number of flagella's increases, the surface area of bacteria increases, and the opportunity for bacterial adhesion increases (Townsley and Yildiz 2015). In this study, the findings corroborated those in the previous findings since the highest median biofilm values were observed at 25 °C (Figure 6(D)). Likewise, light is recognized as a significant variable that directly affects the composition of microbial communities (Piwosz *et al*. 2020). Biofilms are shaped by the interactions between autotrophic and heterotrophic microbes (Schmidt *et al.* 2018). According to this study, the highest median concentration of 1,6 was observed under dark conditions and the lowest optical density of 1,4 was observed under light conditions. This indicated that biofilm formation occurred at a greater rate under dark conditions as compared to light conditions. It has been suggested that the composition of biofilm communities may differ significantly under different light conditions (ambient, dim, and dark) (Pinto *et al*. 2019). The prevalence of slow-growing autotrophs is high in ambient light conditions, since light is the primary source of energy for these organisms (Schmidt *et al*. 2018), while they are low in dark or dim light conditions (Pinto *et al*. 2019). It is likely that, under dim and dark conditions, more rapidly growing heterotrophic bacteria grow in the biofilms. It is possible that this could explain the slightly higher biofilm formation under dark environmental conditions, however, metagenomic studies could provide insight into the microbial communities that emerge under these conditions. Anaerobic and aerobic conditions are known to affect biofilm formation as well as the structure of microbial communities. Earlier studies suggested that low oxygen levels enhance biofilm formation while normal oxygen levels may decrease biofilm formation (Totani *et al*. 2017). The present study, in contrast, has shown a higher biofilm formation rate under aerobic conditions than under anaerobic conditions. However, the difference between the concentration of biofilm under aerobic conditions and anaerobic conditions was statistically

insignificant. The microbial community profiling will provide critical answers for further understanding of the role of aerobic and anaerobic conditions on biofilm formation.

Renner and Weibel (2011) reported that surface roughness of a substratum enhances bacterial adhesion because it provides a larger surface area to attach cells to while reducing the shear forces that bacteria are exposed to while flowing liquids. MPs with rough and charged surfaces are more hydrophilic and are more readily colonized by bacteria than MPs with smooth and inert surfaces, which are more hydrophobic and, therefore, have minimal bacterial colonization (Hossain *et al*. 2019). According to the results obtained (Figure 5), the concentration of biofilm on rough MPs was slightly higher than that on smooth MPs, indicating that surface roughness is helpful for bacterial attachment. Nevertheless, the difference in the amount of biofilm formed on rough versus smooth MPs was not statistically significant. Pinto *et al* (2019) demonstrated the type of surface affects bacterial colonization and community composition more strongly at the beginning of the process than at later stages. It has been demonstrated that bacteria attach more readily to uneven or rough surfaces due to the larger surface area (or contact area) between bacterial cells and their surroundings, which facilitates both bacterial attachment as well as protection from shear forces (Huang *et al*. 2019).

Furthermore, the chemical property of the plastic plays a significant role in the microbial attachment. It has been shown that LDPE, HDPE and PP have unique properties that affect bacterial colonization (Fotopoulou *et al.* 2012). The surfaces of PP are hydrophobic when dry, whereas the surfaces of PE become charged as soon as they are in contact with water (Fotopoulou *et al.* 2012; Hossain *et al*. 2018). Due to differences in their surface properties, HDPE and LDPE exhibit different surface roughness when exposed to stressors (Hossain *et al*. 2018). PP is a methylated form of PE. PP is more hydrophobic than PE, whereas LDPE and HDPE differ chemically depending on the amount of PE chains compacted into the polymer chains (Hossain *et al*. 2018). The observed differences in the amount of biofilm on the different

types of MPs may be explained by these differences. As shown in Figure 6 (A), the results obtained indicated a median value of 1,8 for HDPE and LDPE, while a lower median value (1,7) was found for PP. In spite of this, the difference between amount of biofilm formed on PE and PP was not statistically significant. There is the possibility that surface characteristics may not have a significant impact on the concentration of biofilms on microplastic particles, but rather have a significant impact on the composition of biofilms (Yang *et al*. 2020), thereby influencing the rate of biofilm formation .

In addition, a combination of conditions may influence the concentration of biofilms formed on the three different types of plastics. This study demonstrated that the combined conditions that had the highest impact on biofilm formation differed for each type of plastic as the weeks progressed. Despite the type of plastic, the highest biofilm formation occurred on rough plastics. The combined conditions that produced the highest concentration of biofilms on PP MPs was observed under dark and aerobic conditions (D-A-R) at 35°C and for LDPE MPs was observed on rough microplastics in dark and anaerobic conditions (D-AN-R) at 35°C. Whereas the highest median biofilm concentration on HDPE MPs was observed on rough microplastics under light and aerobic conditions (L-A-R) at 25°C. The biofilms formed on the three types of plastics could consist of bacteria that grow and develop under mesophilic temperature conditions (11°C to 45 °C) (Pandey *et al*. 2015) as growth was observed at 25°C on HDPE and at 35°C on PP and LDPE. The highest biofilm development on PP occurred under aerobic conditions and LDPE under anaerobic conditions in combination with dark conditions at 35°C. This indicates biofilms on PP could be composed of mainly aerobic microorganisms whereas biofilms on LDPE could be made up of mainly anaerobic microorganisms. In contrast, the biofilms present on HDPE preferred a combination of light and aerobic conditions at 25°C. These observations also suggests that even though the type of plastic did not have a significant impact on biofilm concentration, when subjected to a

combination of conditions it may impact the composition of the biofilms as different types of microorganisms require different growth conditions (Koch *et al*. 2016).

Chapter 4: Impact of chlorination and UV treatment on bacterial population dynamics biofilms attached to microplastics

4.1. Introduction

Biofilm formation is an important adaptation of bacteria and one of their major survival strategies, rendering them resistant to antibiotics and disinfectants (Berlanga *et al.*, 2016). Bacterial microcolonies communicate via intercellular signalling or cell-to-cell communication known as "Quorum Sensing" (Brindhadevi *et al.* 2020). Biofilm forming bacteria have certain physiological features, including flagella, fimbriae and pili, that are helpful in the process of biofilm formation (Flemming et al., 2017). Both gram-negative and gram-positive bacteria can form biofilms, but the most common forms reported from MP surfaces are *Pseudomonadaceae*, *Moraxellaceae*, *Enterobacteriaceae and Comamonadaceae* (Khatoon *et al.* 2018; Kelly *et al*. 2021). In wastewater treatment plants (WWTPs), MPs are colonized by a diverse community of microorganisms including pathogenic bacteria such as *Vibrio*, *Campylobacter*, and *Arcobacter* as well antibiotic resistant bacteria (ARB) (Kelly *et al*. 2021).

Characterization of biofilm communities is important as it can provide information on the type of pathogens present and improve tertiary treatment methods to reduce the discharge of harmful pathogens into the environment (Leddy *et al*.2017). DNA based methods have been used to identify and profile the microbial communities present in biofilms (Luhrig *et al*. 2015). DNA-based next-generation sequencing (NGS) can offer more powerful, high-throughput, culture-independent results that provide a more complete, rapid, and accurate identification and characterization as well as functional capability of microbial communities in a mixed consortium, such as in biofilms (Leddy *et al*. 2017).

MPs may play a role in the transport of pathogens through WWTPs and then into the aquatic environment (Hoellein et al., 2017). Biofilms are advantageous to microorganisms because of their excellent ability to retain microorganisms. Microorganisms inside the biofilm are protected from surface detachment, predation, inhibitory, and degrading mechanisms (Rittman., 2018). In this way, biofilms may protect microorganisms and MPs from tertiary treatment methods such as UV irradiation and chlorination during wastewater treatment. The protective mechanism is active for any threat that originates outside the biofilm and reacts with the biofilm. Being located deep inside the biofilm microorganisms are protected from high concentrations of inhibitory substances as the concentration decreases in order to travel into the biofilm by diffusion (Rittman., 2018). This can be a major concern during wastewater treatment because tertiary treatment does not remove all MPs, and treatment mechanisms may have a reduced or no effect on biofilms resulting (Sun et al, 2019; Liu et al, 2019; Rittman, 2018) in the discharge of both microplastics and microplastic-bound microorganisms into the environment.

This chapter aimed to determine the bacterial composition of biofilm attached to three different types of MPs and, the wastewater medium during the five week cultivation under controlled conditions. A comparison was made between the bacterial population of the biofilms from each type of MP with the bacterial population in the wastewater medium. Furthermore, the impact of chlorine and UV tertiary wastewater treatment processes was determined to help to provide information on the effect of common disinfection methods on MP associated biofilms during wastewater treatment.

4.2 Methodology

4.2.1 Experimental Setup

The favorable conditions for biofilm formation determined and described in Chapter 3 were used for the following experiment. The conditions followed included: incubation temperature of 25 ^{o}C, under dark and aerobic conditions. Sampling for microbial analysis was done during Week 1, Week 3 and Week 5.

4.2.2 Profiling of microbial community attached to microplastics and in wastewater medium

Sample Preparation

i. The wastewater medium was collected (2 mL) in Eppendorf tubes and centrifuged at 5000 rpm for 5 mins. The supernatant was discarded, the pellet was resuspended in 1mL dH_2O and centrifuged at 5000 rpm for 5 min. This step was done twice. Thereafter, the supernatant was discarded and resuspended in 1 mL PBS and centrifuged at 5000 rpm for 5 minutes.
ii. MPs (10) were removed from the flasks, rinsed with sterile distilled water, and collected in Eppendorf tubes.

DNA extraction, Sequencing and analysis

Total DNA was extracted using the Phenol-Chloroform extraction method adapted from Awolusi *et al*. (2016). Five hundred microliters of lysis buffer (1 M Tris-HCl; 0.5 M EDTA; 10 % SDS; 5 M) was then added to each tube and vortexed thoroughly for 1 min. The tubes were then placed in water bath at 60^{o}C for 30 mins. The temperature was thereafter raised to 65 ^{o}C, and the tubes were incubated for a further 2 h. A freeze-thaw step was then carried out, by placing the tubes in ethanol-ice slurry for 3 mins and then in a 65 ^{o}C water bath for 3 mins. This was repeated five times. Thereafter 500 µL of a freshly made phenol/chloroform/isoamyl alcohol mix (25:24:1) was added

to the tubes. These tubes were gently mixed by inversion and centrifuged at 10000 rpm for 3 min at 4 °C. The upper aqueous layer was carefully transferred to a fresh set of tubes. Five hundred microliters of chloroform was then added to the tubes. These tubes were gently mixed by inversion and centrifuged at 10000 rpm at for 3 min at 4 °C. The upper aqueous layer was again carefully transferred to a fresh set of tubes, and the DNA was precipitated by the addition of 600 µL of isopropanol and stored at -20 °C overnight. The precipitated genomic DNA was pelleted by centrifugation at 12000 rpm at 4 °C for 20 min, washed with cold 70 % Ethanol and air dried for no longer than 15 mins. The final DNA pellet was stored in 20 µL 1 x TE buffer (pH 7.5) at -20 °C. The purity and quantity of the DNA extracts were analysed by spectrophotometry using the IMPLEN NanoPhotometer. The DNA extracts were then sent for 16S rRNA amplicon sequencing and analysis at Inqaba Biotec (Pretoria, South Africa)

4.2.3 Statistical Analyses

The fastq files were subjected to a DADA2 and a QIIME 2 pipeline. Alpha diversity is applied in analysing complexity of species diversity for a sample through different indices, including Observed-species, Shannon and Simpson. All these indices in our samples were calculated with QIIME . For beta diversity, principal coordinate analysis (PCoA) calculated based on the similarity and distance between the samples, including non-phylogenetic Bray-Curtis distance and phylogenetic-based weighted UniFrac distance also calculated by QIIME. The outputs of the DADA2 pipeline were further subjected to an R studio pipeline to quantify phylogenetic relationship and abundance of species in the sample.

4.2.4. Tertiary Treatment

i. Chlorine treatment

Flasks containing MPs in a wastewater medium after week 5 of incubation were exposed to 12.5 % sodium hypochlorite with a concentration of 5 mg/L for 10, 20, 30 and 60 minutes (WWTP conditions) (USEPA, 1999). The treated MPs were collected at each interval for further analysis.

ii. UV treatment

MPs from flasks containing a wastewater medium after week 5 of incubation were transferred into petri dishes. The petri dishes were illuminated with ultraviolet light emitting 254 nm at a distance of 10 cm from the UV-C source (Lin *et al*. 2020). Exposure was carried out for 10, 15, 30 and 60 minutes. Samples of MPs were collected at each interval for further analysis.

4.2.4 Heterotrophic plate count (HPC)

In order to ascertain the effects of UV radiation and chlorine on biofilms, HPC was performed as per standard methods for water and wastewater testing (APHA, 2011). Approximately 10 MP particles were collected from flasks prior to treatment (control) and thereafter from each treatment and placed in an Eppendorf tube containing 2 mL of PBS. The tubes were then vigorously shaken and vortexed for one minute in order to achieve maximum removal of the biofilms attached to the MPs. Thereafter, about 0.2 mL of biofilm suspension was spread over m-HPC agar and incubated for 48 hours at 35 °C. CFU/MP was determined by counting colonies and by using the following equation:

$$\frac{CFU}{MP} = \frac{number\ of\ colonies\ \times 2\ ml\ (amount\ of\ PBS)}{10}$$

4.3. Results

4.3.1 Bacterial diversity and richness in biofilms and wastewater medium

α-diversity

The profile of the microbial community from both biofilms and the surrounding wastewater medium was determined via 16S rRNA sequencing. The comparison of OTU's for wastewater medium samples across the week (week 0 to week 5) indicated a variation in species richness from Week 0 (WW initial) to Week 5 (WW5) (Table 5). The lowest species richness (1 OTU's) was observed during week 0, whilst the highest species richness (6 OTU's) was observed during week 1, and by week 5, the species richness had decreased to 4 OTU's . A similar trend was observed for biofilms from MPs. For instance, the species richness of biofilm from LDPE was the highest (17 OTU's) at week 1 and the lowest (4 OTU's) was observed at week 5, whereas for PP the highest (18 OTU's), which was also the overall highest species richness, was observed during week 3. The lowest species richness (5 OTU's) of biofilm from PP was also observed during week 5.

The Shannon index of diversity (H') and Simpson index were also determined for the wastewater medium samples and the biofilm samples (Table 5). The Shannon indices of the wastewater medium ranged from 0 to 4 from week 0 to week 5 while for HDPE, LDPE and PP it ranged from 0.88779 to 1.46499, 1.24754 to 2.292 and 1.24963 to 2.51138, respectively. The highest species diversity (H'= 2.51138) in biofilms was observed for PP during week 1, whilst the lowest species diversity (H'=0.88779) was observed for HDPE during week 3. The Simpson indices of the wastewater medium ranged from 1 to 4.18103 from week 0 to week 5 while for HDPE, LDPE and PP, it ranged from 1.5324 to 2.54267, 3.1544 to 7.33374 and 2.74015 to 11.096, respectively. The

highest species diversity (Simpson index = 11.096) in biofilms was observed for PP during week 1 whilst the lowest species diversity (Simpson index = 1.5324) was observed for HDPE during week 3. It was found that PP supported the most diverse (H'= 2.51138 and Simpson index= 11.096), while HDPE supported the least diverse (H'= 0.88779 and Simpson index= 1.5324).

Table 5: Indices of α-diversity of biofilms, including observed species, Simpsons index and Shannon index for MPs and wastewater from week 1 to week 5

Sample	***N.obs** (OTU's)*	*Shannon index (H')*	*Simpson index*
WW initial	1	0	1
WW1	6	1.57084	4.18103
HDPE1	12	1.46499	2.54267
LDPE1	17	2.292	6.70329
PP1	14	2.51138	11.096
HDPE 3	10	0.88779	1.5324
LDPE 3	11	2.17562	7.33374
PP 3	18	2.14752	5.29199
WW5	4	1.11527	2.9952
LDPE 5	4	1.24754	3.11544
PP 5	5	1.24963	2.74015

β-diversity

Eleven rarefaction curves nearly arrived at the plateau phase, which indicated that the results represented the majority of bacterial 16S rRNA sequences in each sample (Figure 10 (A)). Community similarity was evaluated by the Venn diagram, PCoA analysis of all communities and cluster analysis. Principal Coordinate Analysis (PCoA) was used to visualize clusters of biofilms on MPs and the wastewater medium. The Venn diagram (Figure 10 (B)) revealed 762 (0.9%) common OTU's among HDPE, LDPE and PP. It also revealed 955 (1.1%) common OTU's between the MPs and the wastewater medium (Figure 10(B)). The unique OTU's present on HDPE, LDPE and PP were 7609 (8.7%), 12938 (14.8%) and 1176(1.3%), respectively while

unique genera in the wastewater medium was 15207 (17.4%) (Figure 10(B)). The PCoA scatter plot showed a clear distance between the different samples (Figure 10 (C)). The samples that were most similar in abundance were HDPE, LDPE and PP. The weighted UniFrac distance revealed the most similar pairs in abundance were (HDPE1: HDPE3), (PP3:LDPE3), (PP1:LDPE1) and (PP5:WW5) (Figure 10 (C)).

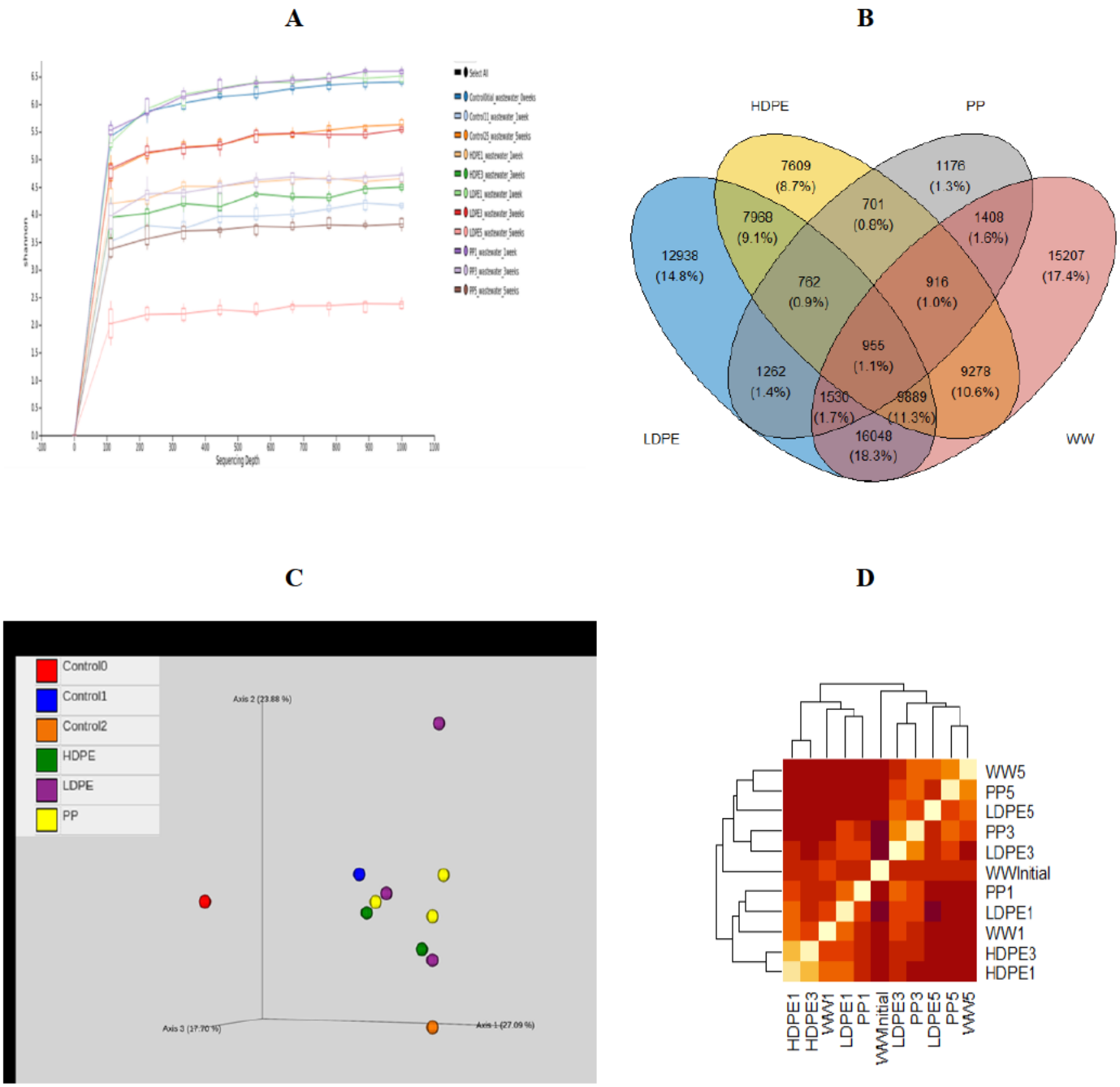

Figure 10: Community similarity analysis in different fillers. (A) Rarefaction curve analysis. (B) Comparison of OTUs from all samples by Venn diagram. (C) PCoA illustration of the samples. (D) Cluster analysis.

4.3.2 Bacterial abundance in biofilms and wastewater medium

At phylum level, the most dominant phyla in the biofilms and wastewater medium during the five weeks were Proteobacteria, Bacteroidetes and Planctomycetes (Figure 11). The abundance of Proteobacteria remained the highest throughout the study period. During week 5, biofilms had the highest abundance of Proteobacteria of 91.28%. The lowest abundance of Proteobacteria of 73.55% was observed during week 1. The abundance of Bacteroidetes in biofilms decreased from week 1 to week 5. For instance, the highest abundance of Bacteroidetes of 15.18 % was observed on week 1 and thereafter decreased to 0.875% by week 5. The abundance of Planctomycetes increased from week 1 to week 3 and thereafter decreased at week 5. The highest abundance of Planctomycetes of 8.73 % in biofilm samples was observed during week 3. The lowest abundance of Planctomycetes of 2.73% was observed during week 5.

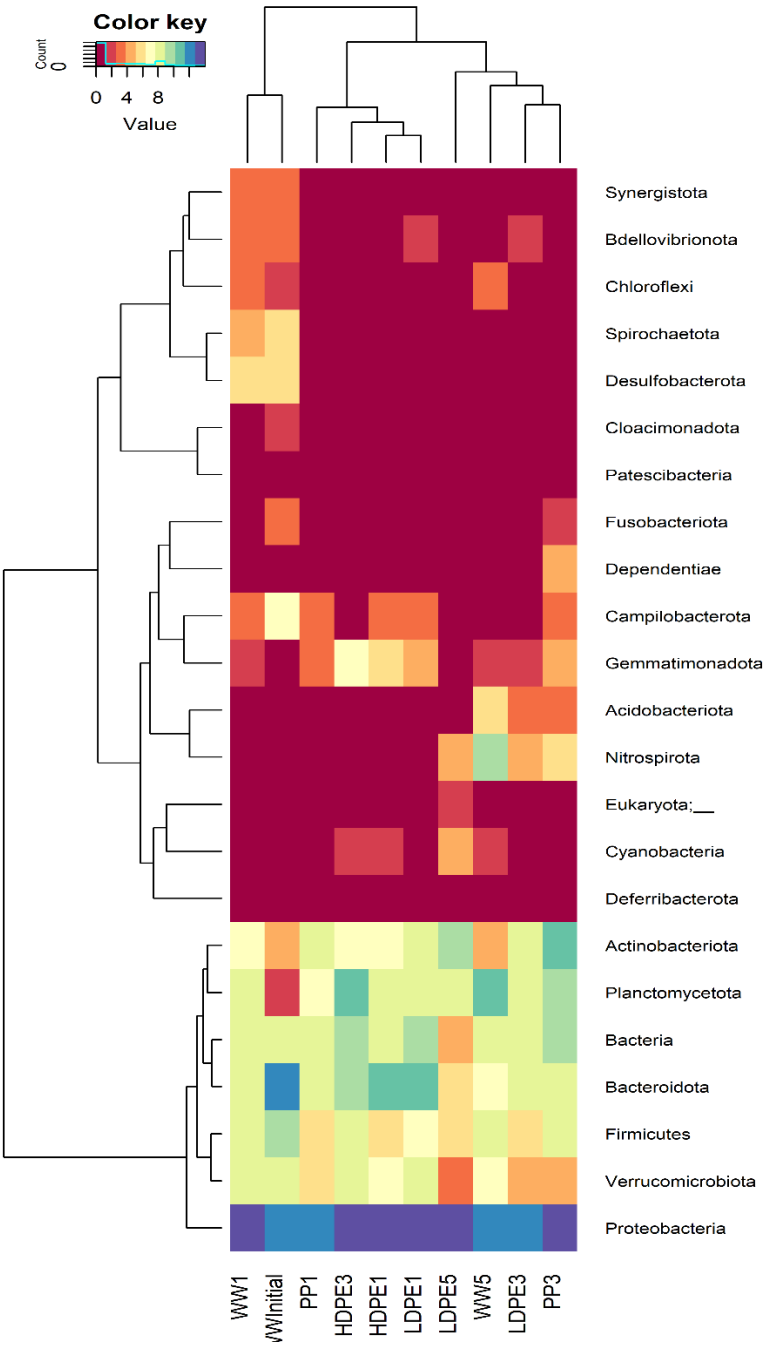

Figure 11: Heatmap of the phyla that were observed in the sample set and the distribution in abundance. The relative values of the bacterial phyla are depicted by colour intensity from red(lowest concentration) to purple (highest concentration).

The bacterial abundance in biofilms on the different types of microplastics

The bacterial population of biofilms varied for each type of microplastic particle. It was observed that *Methylotenera* was the most abundant genus on HDPE during week 1 (34.16%) and week 3 (42.45%) (Figure 12). Whereas for LDPE, the most abundant genus during week 1 was *Hydrogenophaga* with an abundance of 19.07%. However, during week 3 and 5 the most abundant genus was *Methylotenera* (25.39%) and *Rhodanobacter* (14.76%), respectively. The most abundant genus observed on PP during week 1 was *Nevskia* (12.05%) and during week 3 and 5 the most abundant genus was *Methylotenera* (36.85%) and *Rhodanobacter* (32.61%), respectively. Interestingly, it was observed that during week 3, the most abundant genus was *Methylotenera* for the three different types of microplastic particles.

The bacterial abundance in the biofilms and wastewater medium

A variation was observed between the bacterial population in the biofilms and the wastewater medium (Figure 12). For instance, during week 1, the most abundant genus present on the microplastics were *Methylotenera, Hydrogenophaga, Nevskia and Zooglea* whereas the most abundant genus in the wastewater during the same period was C39 (45.25%). Additional genera such as *Planctomyces, Sediminibacterium Polynucleobacter and Prosthecobacter* was also found in wastewater during week 1 . Similarly, during week 5, the bacterial population found in wastewater differed from the population in the biofilms. *Methylibium* and *Rhodanobacter* were the two genera that was present in both biofilms and wastewater medium during week 5. The most abundant genus in wastewater was *Luteimonas* (18.96%) which was not present in biofilm. Additional genera such as *Gemmata , Nitrospira* and *Methylibium* was found in the wastewater medium and not in biofilms.

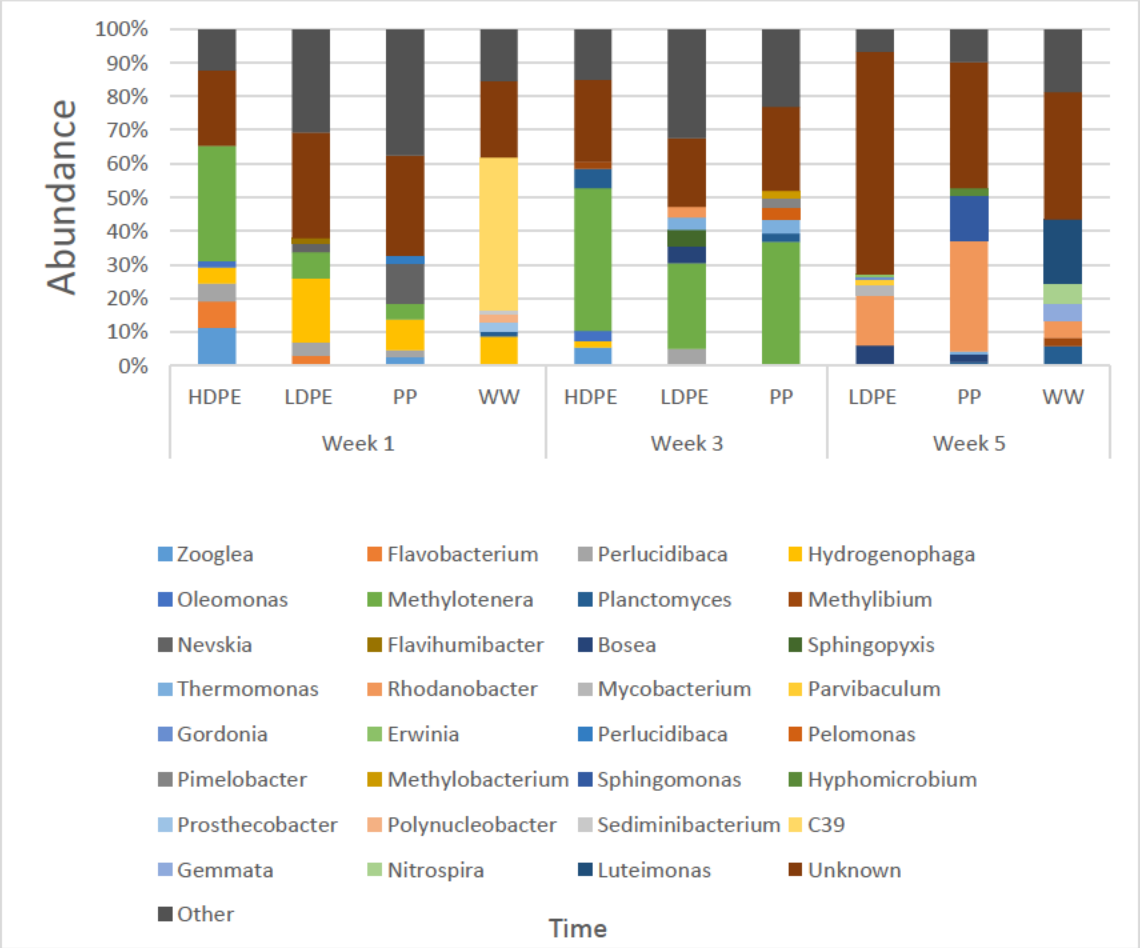

Figure 12: Total population shift at the Genus-level from Week 1 to Week 5 for biofilms on HDPE, LDPE, and PP microplastics and in the wastewater medium

The dominant species abundance in biofilms and the potential pathogenic species

The species abundance varied between the different MPs. *Methylotenera mobilis* was the common specie among the different MPs but the abundance differed for each type of plastic. The abundance of *Methylotenera mobilis* was the highest on HDPE (42.45%) during week 3 while the lowest abundance was on PP (4.58%) during week 1 (Table 6). The unique dominant species among the three 3 MPs observed on LDPE was *Stenotrophomonas acidaminiphila* (1.35%) and *Mycobacterium arupense*

(2.96%). The unique dominant species observed on PP was *Methylobacterium adhaesivum* (1.35%), *Sphingomonas wittichii* (12.89%) and *Rhodanobacter lindaniclasticus* (1.25%).

Table 6:The dominant species detected in biofilms on HDPE, LDPE, and PP microplastics during the study period

HDPE	LDPE	PP
Methylotenera mobilis	*Methylotenera mobilis*	*Methylotenera mobilis*
	Stenotrophomonas acidaminiphila	*Nevskia ramosa*
	Nevskia ramosa	*Thermomonas fusca*
	Thermomonas fusca	*Bosea genosp*
	Bosea genosp	*Methylobacterium adhaesivum*
	Mycobacterium arupense	*Sphingomonas wittichii*
		Rhodanobacter lindaniclasticus

4.3.3 Impact of tertiary treatment on microplastic-bound microorganisms and the wastewater

The results of this study showed (Table 7) that the biofilm on MPs before tertiary treatment (control) contained 15000 CFU/MP, and the number of CFU/MP after 10 minutes of UV treatment had decreased to 2.5 CFU/MP. The colony growth was observed up until 30 minutes after treatment (1 cfu/MP); thereafter, no colony growth was observed. In addition, there was no evidence of colony growth following chlorine treatment (5 mg/L) for 10 to 60 minutes (Table 7), indicating that chlorine treatment for 10 minutes or less is effective in inactivating microbes attached to the microplastics. The percentage inactivation of biofilms was 100% after 60 min of UV treatment while 100% inactivation after chlorine treatment was observed after 10 mins of treatment.

Table 7: Colony forming units per microplastic particle before and after UV and Chlorine treatment of biofilms formed on MPs

Time (Minutes)	UV (cfu/MP)	Chlorine (cfu/MP)
0 (Control)	1500	1500
10	2.5	No growth
15	1.5	No growth
30	1	No growth
60	No growth	No growth

4.4 Discussion

4.4.1 The microbial population in biofilms

Biofilms that form on MPs are made up of diverse bacterial populations (Yasir *et al.*2018). The communities that may develop biofilms under specific conditions on MPs may contain pathogenic microorganisms that could negatively impact the receiving water bodies. In this study we investigated the microbial community structure of the biofilms on MPs substrates in wastewater. Our observations provided evidence that the bacterial community found in biofilms was similar from week 1 to week 5. However, the bacterial communities did differ on each type of plastic. There was also a variation observed in the bacterial population in biofilms and the population found in the wastewater. This could indicate that the microplastic properties could have an influence on the bacterial populations that colonize MPs. In addition, the difference in the bacterial populations in the biofilms and in the wastewater could suggest that there are only certain bacteria that attach to MPs and are able to form biofilms.

According to Zhang *et al*. (2019) Proteobacteria are reported as one of the most dominant biofilm forming phyla. In this study bacteria belonging to Proteobacteria were the most dominant in the biofilms, followed by Bacteroidetes and Planctomycetes (Figure 11). A previous study done by Wu *et al*. (2019) reported the most abundant phyla found on MPs was Proteobacteria and Planctomycetes were much higher in biofilms

formed on MPs than on other natural substrates. Wu *et al*. (2019) also found that the abundance of Bacteroidetes in MP biofilms was lower than in biofilms on other natural substrates. In this study we found Bacteroidetes to be one of the dominant phyla in the biofilms formed on MPs. During the five week period the abundance of Bacteroidetes and Planctomycetes decreased from week 1 to week 5 whereas the abundance of Proteobacteria increased from week 1 to week 5 and the remained the highest throughout the study period. The dominance of Proteobacteria in biofilms could be related to a broad ability of surface colonization, adaptability to changing nutrients and capability to proliferate under oligotrophic conditions (Ivnitsky *et al.* 2010). A study done by Miao *et al.* (2018) reported that in a 3-week culture study, Proteobacteria remained dominant in all biofilm samples followed by Bacteroidetes indicating that irrespective of time Proteobacteria was the dominant phyla in biofilms.

The genera detected in the biofilms were mostly found to be biofilm forming bacteria. The most dominant biofilm forming genera found on MPs were *Methylotenera, Hydrogenophaga, Rhodanobacter* (Figure 11). These genera together with *Flavobacterium* are known to be some of the core bacterial communities associated with biofilms found on MPs (Debroy *et al.* 2021). A study done by Rummel *et al*. (2021) reported that *Methylotenera* was found to be the dominant primary colonizing genus. *Methylotenera sp.* are known as biofilm forming bacteria that can secrete high amounts of EPS (Braun *et al*. 2016; Chen *et al*. 2022). *Methylotenera* was detected on all types of microplastics but was only present during week 1 and week 3. This could indicate that *Methylotenera* is only present during the initial stages of biofilm development (Rummel *et al*. 2021). In previous studies *Methylotenera* was also found to be a dominant primary colonizing genera that may depend on the initial conditioning film. *Methylotenera* may grow slower as a result of the other primary colonisers' dependence and utilization of the conditioning film (Rummel *et al*. 2021; Wang *et al.* 2022; Caruso, 2020) . This could also indicate *Methylotenera* is a weak competitor and could be easily outcompeted. Another abundant genus was *Rhodanobacter.*

Rhodanobacter is an autotroph that is capable of denitrification (Aqeel *et al*. 2016) and was detected during the final week of the study. This correlates with the nutrient study performed in chapter 3 (Figure 5). An increase in nitrite production was observed from week 1 to week 3. The *Rhodanobacter* genus could have been responsible for the denitrification of nitrate. A study done by Zhang et al (2021) similarly found Rhodanobacter as one of the dominant denitrifying genera in biofilms. *Hydrogenophaga* was also found in abundance on all types of microplastics during the first week of the study. This genera are known as aerobic dechlorinating bacterium that is capable of growing autotrophically (Chen *et al*. 2018). *Flavobacterium*, a common biofilm forming genus that was also detected but only during the first week of the study and it favored polyethylene MPs. Its early attachment could indicate the usage of the initial conditioning film (Rummel *et al.* 2021).

There are various types of MPs in the aquatic environment that may have an influence on the microbial attachment and biofilm development (Hansen *et al*. 2021). A previous study reported the MP bacterial community composition was significantly influenced by the polymer type, where polystyrene (PS) presented a distinct bacterial community compared to those of PE and PP (Frère et al., 2018). A study done by Hansen *et al*. (2021)compared the microbial communities on PE, PP and PS plastics and reported a significant difference in the biofilms. The most abundant genera found on the three types of MPs were *Methylotenera, Zooglea, Hydrogenophaga, Rhodanobacter and Nevskia* (Figure 12). The abundance and presence these genera differed among the three types of MPs, but *Methylotenera* remained dominant. It has been widely reported the microbial communities found on plastic debris are different from surrounding free-living bacteria (Nava and Leoni 2021). In this study the bacterial community found in biofilms differed from the population found in wastewater. *Luteimonas, Nitrospira, Gemmata* and C39 are the genera that was found in the wastewater and not in biofilms. The *Nitrospira* found in wastewater during week 5 could be ammonium oxidising *Nitrospira* (comammox). Previous studies have found comammox

Nitrospira able to oxidise both ammonia and nitrite in a single cell (Takahashi et al. 2020; Daims et al. 2015; van Kessel et al. 2015). This could be the reason for the nitrate production that was observed in Chapter 3 (Figure 5).

MPs are known to be a vector for pathogenic microorganisms across large distances in aquatic environments (Boni *et al.* 2021). In this study several different species were detected in the biofilms. The pathogenic species detected were *Mycobacterium arupense* and *Methylobacterium adhaesivum.* Mycobacteria are a potential source of various infectious diseases, in a study done by Wang *et al*. (2021) Mycobacterium abundances found in PVC and PE biofilms were higher than those in sewage samples. In this study *Mycobacterium arupense* was detected in biofilms on LDPE microplastic particles. *Mycobacterium arupense* is a non-tuberculosis mycobacteria (NTM) group of mycobacteria found in environmental samples, such as surface water and soil, however through inhalation and traumatic inclusion *Mycobacterium arupense* can cause mycobacterosis infection (Wang *et al*. 2021). *Methylobacterium* are major inhabitants of aqueous environments that also have a strong biofilm-forming ability (Kovaleva *et al*. 2014; Yano *et al.* 2013). It has been found in potable water supplies and also in hospital tap water (Kovaleva *et al.* 2014). *Methylobacterium spp.* including *Methylobacterium adhaesivum* can cause mild fever and also bacteremia, peritonitis, and pneumonia (Szwetkowski *et al.* 2020). The most dominant species detected was *Methylotenera mobilis*. This was detected on all three types of MPs. Other species such as *Bosea genosp, Thermomonas fusca, Stenotrophomonas acidaminiphila* and *Rhodanobacter lindaniclasticus* are known for their nitrogen fixing abilities were also detected in biofilms (Estendorfer *et al.* 2020; Chen *et al*. 2020). *Rhodanobacter lindaniclasticus* and *Sphingomonas wittichii* are known to degrade toxic chemicals including pesticides and herbicides (Kumar 2018 ; Miller *et al.* 2010)

Pathogens colonize MPs during their journey in the wastewater treatment process. WWTP are designed to remove pathogens from wastewater however some pathogenic species were detected on MPs in rivers downstream of the WWTP (McCormick *et al*. 2014; McCormick *et al*. 2016). These pathogenic species entering rivers could negatively impact organisms in river as well as humans that use the river water (Kelly *et al*. 2021). Therefore, it is important that treatment processes ensure the proper removal of these MPs as well as proper disinfection measures during the final stages of tertiary treatments. Disinfection methods need to effectively deactivate pathogens before wastewater is discharged into receiving water bodies.

4.4.2 The impact of UV and Chlorine tertiary treatment on biofilms

MPs have been detected in wastewater treatment plant effluent, and they have been demonstrated to serve as habitats and transport vehicles for microbial pathogens. This highlights the importance of tertiary treatment in the process of inactivating attached microbes (Kaur *et al.* 2021). Biofilms resist tertiary treatment primarily by forming thick matrices, and UV radiation can only penetrate the top layer of microbial cells (de Carvalho 2017). UV-C radiation reacts with DNA and RNA hindering replication and transcription processes and therefore inactivates the growth of microorganisms (Rosario *et al.* 2021). It has been suggested that the effectiveness of UV radiation treatment is also influenced by the age of the biofilm as well as the thickness and EPS content in biofilms (Argyraki *et al*. 2017; Luo *et al*. 2022). Despite this, several studies have demonstrated the effectiveness of ultraviolet light for inactivating microbial cells in biofilms. Based on the findings of this study, bacteria were able to survive up to 30 minutes (Table 6) after UV light exposure under laboratory conditions. However, no bacterial growth was observed thereafter. This indicates that the UV light penetrated through the layers of biofilm and was able to inactivate the microbial cells after 30 minutes of exposure. Additionally, Harada and Nascimento (2021) reported the maximum reduction in *B. cereus* biofilm population on PP occurred after 30 minutes of UV-C treatment. Gora *et al.* (2019) reported that UVC light at 265 nm could inactivate biofilm-bound *P.*

aeruginosa cells; however, biofilm-bound cells proved more resistant to inactivation than planktonic cells, suggesting that biofilms provide some level of protection to the cells in them. The chlorination process is a common treatment used in wastewater treatment plants due to its prolonged germicidal ability and ability to affect biofilm formation at various stages (Ibekwe and Murinda 2019). The inactivation efficiency of chlorine for biofilms depends on its activation strength and diffusibility (Shen *et al*. 2017). Chlorine penetrates and damages cell membranes, releasing proteins and nucleic acids, and inhibits enzyme activity thus killing bacteria (Kelkar *et al*. 2019). It also decreases biofilm hydrophobicity and adhesion by reacting with proteins and polysaccharides (Luo *et al*. 2022). Buse *et a*l. (2019) reported that *L. pneumophila* biofilm was inactivated on PVC surfaces using free chlorine after 30 min of exposure. In this study, no bacterial growth was observed from biofilms (Table 7) after 10 minutes of exposure to chlorine. Harada and Nascimento (2021) also reported a reduction in B. *cereus* biofilm populations on PP after 15 minutes of treatment with sodium hypochlorite. Additionally, it was observed that UV treatment requires a longer duration of exposure time than chlorination for inactivating biofilms in the study. Similarly, Harada and Nascimento (2021) reported that UV-C had less effectiveness against biofilms than sodium hypochlorite, since UV-C treatment methods require long periods of exposure in order to achieve high biofilm reduction. UV light is a rapid, low maintenance and environmentally safe disinfection method compared to chlorine however UV is limited because of photoreactivation and dark repair of bacteria (Wang *et al*. 2021). Chlorine is also limited due to its inability to inactivate some chlorine-resistant bacteria but residual chlorine in water distribution systems after treatment provide residual protection and prevents potential regrowth of bacteria (Wang *et al.* 2021; Li *et al.* 2018).

Chapter 5: Conclusions and Recommendations

5.1 Conclusions

This study examined the factors that may contribute to microbial attachment and biofilm formation on the surface of different MPs (HDPE, LDPE, and PP) in wastewater. The major conclusions from the study are highlighted below. During the five-week study period, three distinct stages of biofilm development were observed. The maximum concentration of biofilms on MPs was observed after three weeks, after which the concentration decreased. Environmental conditions and nutrient availability of the media played a pivotal role in the development and growth of biofilms. The greatest biofilm formation occurred when the incubation temperature was 25 °C, under dark and aerobic conditions, suggesting that these conditions may be most favorable for biofilm formation by biofilm forming bacteria. Also, it was found that the physicochemical properties of the microplastic particles influenced the concentration of biofilms on the microplastic particles. Biofilm formation on polyethylene (HDPE and LDPE) MPs was greater than that on polypropylene (PP) MPs. In addition, the surface texture of the MPs affected biofilm formation. In comparison to smooth MPs, rough MPs had a higher concentration of biofilm attachment.

The bacterial community diversity of the biofilms from MPs were observed to be more diverse and richer that the bacterial community in the wastewater medium. The bacterial community detected in biofilms remained similar during the 5-week study period. However, their dominance varied across the growth period. The dominant phyla detected in biofilms were Proteobacteria, followed by Bacteroidetes and Planctomycetes. Proteobacteria was the most abundant phyla throughout the study period. However, the abundance decreased from 15.18% to 0.875% and 8.73% to 2.73% for Bacteroidetes and Planctomyces respectively, from week 1 to week 5. The type of plastic did have an influence on the microbial community

that was attached to each type of microplastic particle. The most dominant biofilm forming genera found on microplastics were *Methylotenera, Hydrogenophaga, Rhodanobacter*. These genera detected in the biofilms did differ in abundance for each type of plastic. The species abundance also varied amongst the different MPs. *Methylotenera mobilis* was found to be the most common specie amongst the three types of MPs. Pathogenic species such as *Mycobacterium arupense* and *Methylobacterium adhaesivum* were also detected in abundance on LDPE and PP MPs.

This study also found that UV and chlorine treatments were both effective at inactivating attached biofilms under optimal conditions. A 10-minute contact time was required for chlorine treatment whereas a 30-minute exposure to UV light was sufficient to inactivate biofilms under controlled conditions.

5.2 Observations and Recommendations

A future study could examine how wastewater treatment operational conditions impact biofilm formation in a full-scale environment. In addition to the batch culture conducted in this study, it is recommended that the microbial composition of biofilms be investigated under continuous culture conditions in order to gain an understanding of biofilm attachment under continuous nutritional supply. Due to the presence of pathogenic species in biofilms, further studies should focus on identifying pathogenic species in biofilms formed on microplastics. Furthermore, it is also necessary to investigate the antibiotic resistance of microorganisms within biofilms as it could be potential carriers of ARBs in the environment. Despite the effectiveness of UV and chlorine treatment in deactivating biofilms, it is still possible for biofilms to regrow or reform following tertiary treatment and the use of HPC-based detection alone can be misleading. In order to gain a deeper understanding of the efficacy of the tertiary methods used in this study, additional research using alternative methods of microbial detection is therefore recommended.

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Appendix A: Scanning Electron Microscopy Images

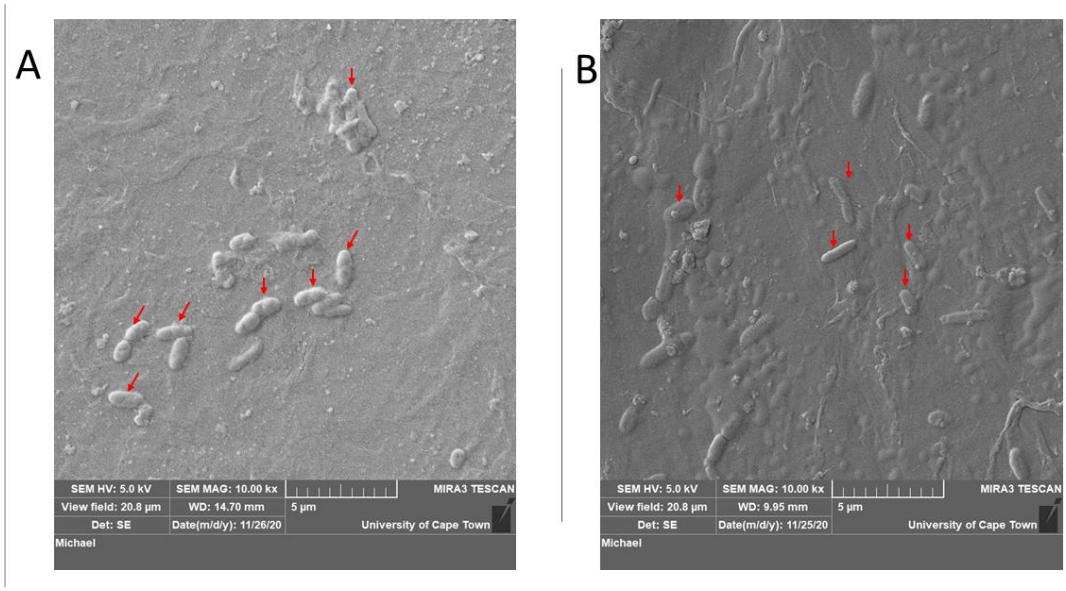

Figure A1: SEM images to show bacterial attachment to smooth (A) and rough (B) LDPE.

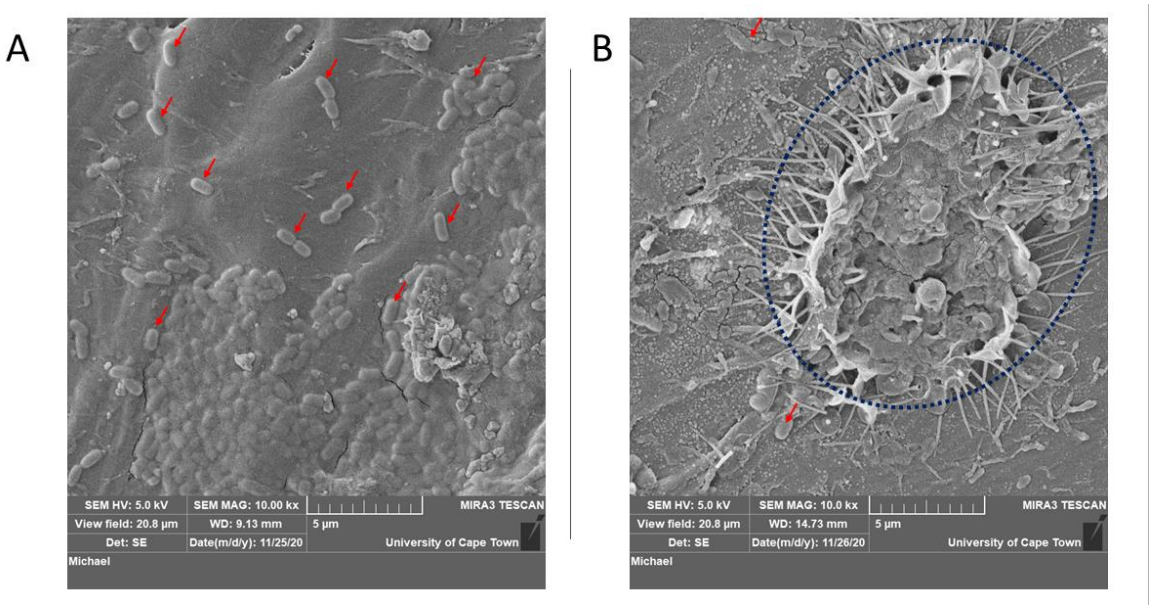

Figure A2: SEM images to show bacterial attachment to smooth (A) and rough (B) HDPE