EXPERIMENTAL INVESTIGATION ON MICROPLASTICS BIOFOULING RATE IN AN AQUATIC SYSTEM

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Abstract

Microplastics (MPs), small plastic particles under 5 millimeters, are a growing environmental concern in freshwater and marine ecosystems. They originate from numerous sources, can easily move due to their small size, and hold the potential to transport pollutants through their movements in marine and freshwater ecosystems. Hence, formulating the distribution and movement of these microplastics within aquatic environments is very important. However, MP distribution and mobility in water are complex processes which depend on many environmental and physical factors. One of the factors contributing to the dynamic nature of microplastics is biofouling. Biofouling, a process where MPs undergo surface changes and accumulate microorganisms, is a key factor influenced by factors like size, shape, and environmental conditions. Water quality in Lake Ontario is impacted by urban and wastewater discharge and across the lake MPs are reported at different levels. The wastewater effluent influences biofilm formation on MPs and in turn, affects their movement in the lake. Despite increased research on microplastic biofouling in freshwater, a significant research gap still exists especially in experimental works. To address this gap, in this study, a series of lab-based experiments, simulating Lake Ontario conditions, were conducted to analyze bacterial growth and biofilm formation on different microplastic sizes. The study investigated factors such as bacteria type, nutrient availability, shaking speed, microplastic size, and flow rate on MP biofouling formation and growth.

This study highlights the significance of biofouling on microplastics, focusing on how bacterial competition, nutrient supply, and the rate of water flow affect the formation of biofilms. This understanding is crucial for addressing hydro-environmental issues related to biofouling and microplastics, providing insight into mitigation measures.

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Table of Contents

Abstract ii
Acknowledgements iii
Table of Contents iv
List of Figures vii
List of Tables viii
Chapter One: Introduction and literature review 1
1.1 Introduction 1
1.2 Research approach and thesis layout 3
1.3 Biofouling composition and process 5
1.3.1 Conditioning film 5
1.3.2 Colonization by pioneer species 7
1.3.3 Colonization by other microorganisms 7
1.3.4 Accumulation 7
1.4 Effective parameters on MPs' biofouling 8
1.4.1 MPs' characteristics 8
1.4.1.1 Effects of MPs' physical properties on biofouling 17
1.4.1.2 Effects of roughness, surface energy and hydrophobicity on biofouling 19
1.4.2 Environmental parameters 20
1.4.2.1 Effects of water parameters (depth, light, temperature, DO, ORP) on biofouling 25
1.4.2.2 Effects of season and geographic location on biofouling 26
1.4.2.3 Effects of nutrition levels on biofouling 26
1.4.2.4 Effects of type of environments on biofouling 26
1.5 Biofouling effects on MPs' vertical transportation and reviewed studies 27
1.6 Wastewater effects on biofouling 28
1.7 Research gaps 30
1.8 Research motivation 31
Chapter Two: Methodology 33
2.1 Materials 35
2.1.1 Chemicals and reagents 35
2.1.1.1 Synthetic water preparation 35
2.1.1.2 Nutrients and natural organic matter 35

2.1.1.3 Media preparation 35
2.1.1.4 DNA test 35
2.1.1.5 Dinitro salicylic acid (DNS) test 35
2.1.1.6 COD, Nitrogen- ammonia test, and total phosphorous test 36
2.1.1.7 Protein content measurement 36
2.2 Methods 36
2.2.1 Synthetic water preparation and adding nutrients 36
2.2.2 Isolating bacteria from effluent 37
2.2.3 Growing bacteria in the nutrient broth (NB) and plate 39
2.2.4 Bacteria in ambient water 41
2.2.5 Deoxyribonucleic acid (DNA) test to identify the type of bacteria 41
2.2.6 Setting-up the continuous system 43
2.2.6.1 Setup description 43
2.2.7 Optical density test 45
2.2.8 Dinitrosalicylic acid (DNS) test 46
2.2.9 Evaluating the function of systems 48
2.2.9.1 Optical density 48
2.2.9.2 Dinitrosalicylic acid (DNS) test 48
2.2.9.3 Chemical oxygen demand (COD) 48
2.2.9.4 Nitrogen- ammonia 49
2.2.9.5 Total phosphorous 50
2.2.9.6 Protein content 50
2.2.9.7 Light microscopy 52
2.2.10 Scanning electron microscopy (SEM) 52
Chapter Three: Results and discussion 53
3.1 Kinetics results and glucose consumption by bacteria in ambient water 54
3.2 Effects of extra nitrogen source 56
3.3 Bacterial identification 58
3.4 Bacterial growth in ambient water with different combinations of bacteria 59
3.5 Microscope study on the effects of MPs' size on biofilm formation 61
3.6 Effects of flow rate on bacterial growth in the continuous systems 63
3.7 Microscope study on the effects of flow rate on biofilm formation 64
3.8 SEM results on MPs surfaces before and after biofouling 66
3.9 Protein content in the ambient water 70

Chapter Four: Conclusion and future work 72
4.1 Conclusion 72
4.2 Limitation of these Experiments 73
4.3 Future recommendations 74
Bibliography 78

List of Figures

Figure 1-1 The formation and composition of the conditioning film on plastic surfaces..... 6

Figure 1-2 MPs biofouling process steps and effects of MPs characteristics on biofouling .. 8

Figure 1-3 Effects of environmental parameters on the MPs' biofilm growth..... 20

Figure 1-4 MPs enter the aquatic system from different sources and then keep moving up and down by biofouling and defouling process 28

Figure 2-1 Wastewater treatment plant location and approximate sampling area..... 33

Figure 2-2 Preparing 1L synthetic water based on USEPA 2002 method..... 37

Figure 2-3 Representation of serial dilution method for bacterial isolation 38

Figure 2-4 DNA isolation 42

Figure 2-5 Schematic design of a continuous system..... 44

Figure 2-6 Setting up the continuous systems 45

Figure 3-1 (a) Optical density and (b) Dinitrosalicylic acid results in ambient water including bacteria..... 56

Figure 3-2 Effects of extra nitrogen source on bacterial growth -a) wastewater 1, b) wastewater 2, c) wastewater 5 58

Figure 3-3 Optical density results for different combinations of bacteria in the ambient water including a) large MPs and b) small MPs..... 60

Figure 3-4 Comparing surfaces of virgin MPs (right) and biofouled MPs surface in the batch systems after two weeks: a) large MPs: no specific changes can be seen on the surface and b) small MPs: surface changes could be the result of biofilm formation. 62

Figure 3-5 Comparing ambient water at t=0 and t=two weeks in the batch systems) large MPs: without any bacterial colonization and b) small MPs: colonization of bacteria in the ambient water could be the result of biofilm formation in the system 62

Figure 3-6 Comparing OD results in three continuous systems 63

Figure 3-7 (a) MP's surface changes: no specific changes in the first system, but MP's surface in two other systems changed, possibly because of biofouling. and (b) Ambient water of three continuous systems after 10 days shows more bacterial colonization in 2nd and 3rd system which could be the result of biofouling..... 65

Figure 3-8 SEM results on MPs surfaces before and after biofouling (1000X). a) shows MP's surface without any biofilm. There are some changes in MP's surface compared to virgin MP, while others remain the same. c) and d) MPs' surface is completely covered by biofilm 68

Figure 3-9 SEM results on MPs surfaces before and after biofouling (5000X magnification). a) shows MP's surface without any biofilm. There are some changes in MP's surface compared to virgin MP, while others remain the same. c) and d) MPs' surface is completely covered by biofilm..... 69

Figure 3-10 Protein content in three systems with three different flowrates (1st: 65 mL/min; 2nd: 45 mL/min; 3rd: 60 mL/min) versus time 70

List of Tables

Table 1-1 Effective characteristics of MPs on the biofouling rate 9
Table 1-2 Effective environmental factors on the MPs' biofouling rate 21
Table 3-1 Result of DNA tests..... 59

List of Abbreviations

CF	Conditioning Film
CFU	Colony-Forming Unit
Chl-a	Chlorophyll a
COD	Chemical Oxygen Demand
DNA	Deoxyribonucleic acid
DNS	Dinitrosalicylic acid
DOM	Dissolved Organic Matter
DOM	Dissolved Oxygen
EPS	Extracellular Polymeric Substances
FIB	Focused Ion Beam
HDPE	High-Density Polyethylene
LDPE	Low-Density Polyethylene
MP	Microplastic
NB	Nutrient Broth
NOM	Natural Organic Matter
OD	Optical Density
ORP	Oxidation-Reduction Potential
PE	Polyethylene
PET	Polyethylene terephthalate
PP	Polypropylene
PS	Polystyrene

PVC	Polyvinyl Chloride
SCOD	Soluble Chemical Oxygen Demand
TEM	Transmission Electron Microscopy
USEPA	United States Environmental Protection Agency
UV	Ultraviolet
UV-VIS	Ultraviolet-Visible
WRRF	Water Resource Recovery Facility

Chapter One: Introduction and literature review

1.1 Introduction

Microplastics (MPs), small plastic particles less than 5mm in size, have become a major environmental concern due to their widespread presence in marine and freshwater ecosystems. Biofouling, the process by which microorganisms colonize and accumulate on microplastic surfaces, is a phenomenon that can significantly affect the fate, transport, and ecological impacts of microplastics in aquatic environments.

Research on microplastics biofouling has gained significant attention in recent years as scientists strive to understand the mechanisms and implications of this process. Here is a brief background on the key topics on microplastics biofouling available in the literature:

- Biofouling mechanisms: Microplastics can be fouled by a variety of microorganisms, including bacteria, algae, fungi, and other microorganisms. Biofouling can occur through different mechanisms, such as adhesion, colonization, growth, and biofilm formation on microplastic surfaces. The physicochemical properties of microplastics, such as their surface roughness, charge, and hydrophobicity, can influence biofouling processes (Zhai et al., 2023).
- Factors influencing microplastics biofouling: several factors can influence the extent and nature of microplastics biofouling. These include the type of microplastic material, water chemistry, temperature, salinity, nutrient availability, and exposure duration. For example, microplastics made of different polymers may have varying levels of biofouling due to differences in surface properties and biodegradability (Mendrik et al., 2023).

- Ecological implications: Biofouling of microplastics can have ecological implications at multiple levels. First, biofouling can alter the physical and chemical properties of microplastics, such as their size, shape, buoyancy, and degradation rates, which can affect their transport, fate, and persistence in the environment. Second, biofouled microplastics can carry microorganisms and pollutants from one place to another, potentially influencing the spread of invasive species and pathogens. Third, biofouling on microplastics can affect their ingestion, trophic transfer, and toxicity to aquatic organisms, with potential implications for food web dynamics and ecosystem health (Khalid et al., 2021).
- Analytical methods: Research on microplastics biofouling requires specialized analytical methods for quantifying and characterizing biofouling communities on microplastics. These can include techniques such as microscopy, deoxyribonucleic acid (DNA)-based methods (e.g., polymerase chain reaction, high-throughput sequencing), and biochemical assays to identify and quantify microorganisms, their biofilms, and associated extracellular polymeric substances on microplastic surfaces (Khalid et al., 2021).
- Mitigation strategies: Understanding the mechanisms and implications of microplastics biofouling can inform the development of mitigation strategies. For example, modifying the surface properties of microplastics to reduce biofouling, such as through surface coatings or modifications, has been proposed as a potential approach. However, the effectiveness, feasibility, and potential unintended consequences of such strategies need further research and evaluation (Golgoli et al. 2021).

Overall, research on microplastics biofouling is an important and rapidly evolving field that seeks to understand the complex interactions between microplastics and microorganisms in aquatic ecosystems. It has implications for our understanding of microplastics fate, transport, and ecological impacts, as well as for the development of mitigation strategies to reduce the environmental risks associated with microplastics pollution. In the following sections, a comprehensive review has been done on the literature in this study area.

1.2 Research approach and thesis layout

The first step of this research is to conduct a comprehensive literature review which is presented in Chapter 1. This chapter discusses existing literature on microplastic pollution, biofilm formation, and their ecological consequences. This chapter highlights relevant studies related to bacterial growth, water quality, and biofouling in aquatic environments. In Chapter 2, the gaps in knowledge that the current study aims to address are also identified.

The methodology is presented in Chapter 2 and encompasses a systematic approach to comprehensively investigate the biofouling process of microplastics in conditions near wastewater outlets in Lake Ontario. Synthetic water composition was designed based on Lake Ontario's alkalinity, utilizing components like deionized water, $NaHCO_3$, $CaSO_4$, $MgSO_4$, and KCl. The rationale behind nutrient choices, including glucose, ammonium chloride, diammonium phosphate, and Suwannee River NOM (natural organic matters), was to maintain a balanced carbon-nitrogen-phosphorus (C/N/P) ratio that encourages optimal bacterial growth while inhibiting excessive EPS (extracellular polymeric substances) production. Bacteria were isolated from wastewater effluent using a serial dilution method, providing controlled separation and isolation of distinct bacterial species. These bacteria were cultivated in nutrient broth and agar plates to observe growth patterns in both liquid and solid media. The process of growing bacteria

in ambient water involved testing various combinations of nutrient sources and species selection criteria. DNA isolation techniques were employed to identify the type of bacteria, which helped to better understand the microbial composition. The optical density test served to monitor bacterial growth and kinetics, while the DNS (dinitrosalicylic acid) test quantified reducing sugar production and carbon source utilization efficiency. A continuous system was set up to evaluate flow rate effects, and system functionality was evaluated through OD (optical density), DNS, and water quality measurements. Protein content measurement served as an indicator for biofilm characterization. Additionally, light microscopy and SEM (scanning electron microscopy) were utilized to assess biofilm morphology, concluding a comprehensive research approach shedding light on microplastics' interactions with bacterial communities in aquatic environments influenced by wastewater outlets.

The results that are presented in Chapter 3 reveal valuable insights into the biofouling process of microplastics in conditions near wastewater outlets in Lake Ontario. Optical density measurements and DNS results provided a dynamic view of bacterial growth and carbon source utilization, elucidating the intricate relationship between microbial communities and microplastics. Water quality measurements, encompassing COD (chemical oxygen demand), ammonia, and phosphorus levels, demonstrated the interplay between nutrient availability and bacterial activity in the studied systems. The correlation between protein content measurements and biofilm formation further underscored the role of bacterial activity in biofilm development. Microscopy findings presented vivid images of biofilm formations on microplastics, offering a visual representation of the complex microbial interactions taking place in the aquatic environment. Chapter 4 presents the interpretation, discussion of the results, research gaps, and future directions.

1.3 Biofouling composition and process

In the aquatic environment, all immersed surfaces (natural or artificial) are rapidly colonized by a succession of organisms, the outcome being known as 'biofouling'.

In biofouling, organic matter and organisms accumulate over time. Plastic's hydrophobicity causes organic substances to quickly adsorb, which results in conditioning films (CFs) (Artham et al. 2009a, Zettler, Mincer, and Amaral-Zettler 2013). CFs are surface coatings formed by the absorption of biomolecules from the surrounding environment that can modify the material-specific surface properties and precede the attachment of microorganisms. CF often allows irreversible adherence of bacteria to the substratum. Hence, such molecules may play a key role in the initiation of biofilm colonization (Bradshaw et al., 1997) and soon after, bacteria colonize and microalgae grow, sharing space with invertebrates (Kaiser, Kowalski, and Waniek 2017a). Consequently, the biofouling process consists of the following stages.

1.3.1 Conditioning film

A layer of dissolved organic matter (DOM) that is immediately absorbed on the immersed surfaces in the water is the conditioning film and it appears before colonization by microbes on these surfaces. The DOM adsorption on the solid surfaces takes place from minutes to hours and gradually provides a layer of organic matter on the surface. This first conditioning film or, when referring to particles, the so-called eco-corona[1], can alter original substratum surface properties such as roughness, and surface energy. Thus, it has major impacts on biofilm formation and may alter the fate, stability, and ecotoxicological potential of MP particles (Bradshaw et al. 1997; Artham et al. 2009a; Rummel et al. 2021).

[1] The microenvironment of plastics' surface area, known as ecological corona (eco-corona), is characterized by the complex and dynamic adsorption of extracellular organic molecules, leading to a surface organic layer (corona), which affects the plastics' behaviour and their interactions with organisms (Pathan et al., 2020)

Conditioning films can consist of many organic compounds such as glycoproteins, lipids, nucleic acids, ions, polysaccharides, proteins, aromatic amino acids, humic substances, absorbed carbohydrates like uronic, pyruvate, sulfate, proteins, exopolysaccharides, etc. (Bhagwat et al., 2021a), as shown in Figure 1-1.

These biomolecules are mainly the products of the metabolic activities of aquatic organisms that include EPS, exoproteome (signalling molecules), etc. Due to the complex nature of these biomolecules, the formation of CFs can potentially influence the physicochemical properties of materials including the surface tension, charge density, and roughness. Furthermore, CFs can alter the chemical composition of the substratum surface and other physicochemical properties such as wettability and free energy(Bhagwat et al., 2021b). Thus, the subsequent microbial colonization is influenced by the composition of the conditioning film, the nature of the substratum, the nature of the aquatic phase, and the species composition of the microbial community in the aquatic phase (Lewis, 1998a).

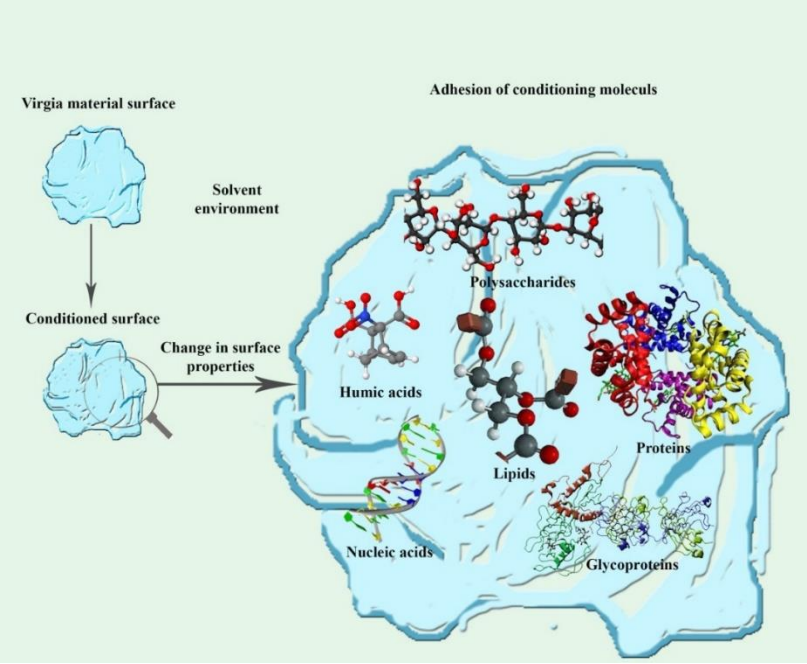


Figure 1-1 The formation and composition of the conditioning film on plastic surfaces

1.3.2 Colonization by pioneer species

After conditioning film formation, small, rod-shaped bacteria attach to it within several hours. As long as bacteria not secreting extracellular adhesive polysaccharides, the initial attachment is weak and reversible (adsorption). Afterwards, primary colonizers consume nutrients and synthesize new cellular and extracellular material on the surfaces. In the next step, additional colonists, such as filamentous bacteria, diatoms, other microalgae, and protozoa, are also present (Lewis, 1998a)

1.3.3 Colonization by other microorganisms

The last and longest phase of fouling colonization is settlement, attachment, and growth of multicellular organisms and after the surface is exposed for the first time, it begins several days or weeks later. In the absence of any antifouling agent, the build-up of organisms will proceed until most of the bare surface is occupied. For fouling communities to develop, species must be recruited onto the surface, compete with resident organisms, and be disturbed by predation or the environment. A macrofouler's initial period of recruitment is defined by a rapid growth rate and a short maturation period. The latter ones are poor recruiters, but the fact that they have a large body size, and a long lifespan makes them competitively successful. Interaction between these organisms that build up the structure and composition of the biofouling community includes facilitation, inhibition, and tolerance. In facilitation, species that reside there enhance the chances of colonization of the next species. In inhabitation, invading species are resisted, and tolerance occurs when resident and colonizing species interact (Lewis, 1998b).

1.3.4 Accumulation

Followed by colonization, the final stage of the biofouling process is the accumulation phase which begins. The microbial film provided after the colonization stage is very different from the original surface. It is highly adsorptive which is partially due to its polyelectrolyte nature and can collect

quantities of particulates and cellular debris (including particles, dead cells, detritus, etc.). In addition, the polysaccharide secretions of microorganisms possess a net negative charge and are capable of chelating inorganic ions from the passing fluid. Fouling deposits are mainly composed of microorganisms and their remains; however, organic secretions, trapped detritus, and inorganic precipitate comprise the majority (Little and Depalma 2013)

1.4 Effective parameters on MPs' biofouling

Density changes of the MPs by biofouling related to characteristics of MPs and environmental factors. In the following, effective parameters in these two categories and their impacts on biofouling have been discussed.

1.4.1 MPs' characteristics

MPs' physical properties (size, shape, type of polymers, and density), surface energy, roughness, and hydrophobicity which have been discussed in the following subsections are from characteristics of MPs that influence biofouling. The effects of these parameters on the biofouling rate have been investigated. Table 1-1 briefly summarizes these studies. MPs biofouling process steps and effects of MPs characteristics on biofouling have been shown in Figure 1-2.

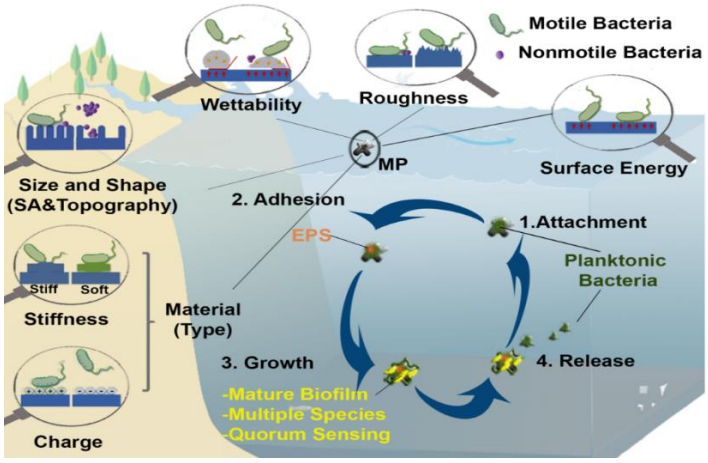


Figure 1-2 MPs biofouling process steps and effects of MPs characteristics on biofouling

Table 1-1 Effective characteristics of MPs on the biofouling rate

Parameter	No.	Type of Study	Study approach	Limitation	Remark	Ref.
1. Size, Shape, Type, and Density	1.1	FW[1]	• Providing a first-order approximation of the load of plastic debris in surface waters of the open ocean • Determining the size distribution of floating plastic debris on the surface of the open ocean, mostly accumulating in the convergence • Data sources: Samples collected on a circumnavigation cruise (Malaspina 2010 expedition), and available data from other studies	• Below 5 mm, the observed size distribution diverged from that expected from the model • Surface losses by sinking are unaccounted for in the model	• The size distribution showed a peak in abundance of fragments around 2 mm and a pronounced gap below 1 mm • Size distribution of floating plastic debris pointed at important size-selective sinks removing millimeter-sized fragments of floating plastic on a large scale • The abundance of MP particles in deep sediments showed a mechanism for the vertical transport of plastic particles, such as biofouling or ingestion.	(Cózar et al. 2014)
	1.2	FW	• Study of the effects of season, location, and plastic substrate type on the structure and diversity of PE, PS, and PP particles • Site location: the coastal and offshore Northern European waters	• The exposure experiment was done in 6-weeks and three seasons, winter, spring, and summer	• Microbial community variation on different polymer types was observed, but communities were primarily dominated by Cyanobacteria.	(Oberbeckmann et al)
	1.3	FW	• Study of the size and composition of floating marine debris as a function of	• Observations have been limited close to the main source of marine	• Size is important in determining the time it takes for biofouling because relative surface area increases as items get smaller	(Ryan, 2015)(, 2015)

[1] FW: Fieldwork, Exp.: Experimental, M: Modeling

	Parameter	No.	Type of Study	Study approach	Limitation	Remark	Ref.
				distance from major litter sources. • Data sources: Floating marine debris recorded during four research cruises from Cape Town aboard the R.V. S.A. Agulhas II: to Tristan da Cunha and Gough Island (40°S, 10°W)	litter and all of them were conducted in the mid-afternoon, and docked in the early morning • only prominent epibionts were visible, and only litter items close to the ship could be examined	• Item shape is important because, for a given volume, surface area to volume ratios are lowest for spherical items and increase as items become elongated, flattened, or have complex shapes	
2.	Size, Shape, Type, and Density	1.4	FW	• Evaluating the effects of plastic particles' size and their surface area on the biofouling • Site location: False Bay Yacht Club in Simon's Town near Cape Town, South Africa	• The time of the experiment was limited from October to December • The samples were fixed 10 cm below the water surface • The experiment was performed in the sheltered environment of a marina where the proximity of other moored items may have promoted rapid fouling.	• The maximum height of the fouling cover recorded during the study was 160 mm on a 1-mm thick macro-HDPE sample at Week 8 and on a 0.5-mm thick, macro-HDPE sample at Week 12. For LDPE samples, the highest growth observed on micro and meso samples was 110 mm at Week 12 and 100 mm at Week 10, respectively. The highest growth found on 0.1-mm thick LDPE was 125 mm on a macro sample at Week 8. • There was no evidence of selection preferences based on sample size or material thickness by any of the most common foulants. • By the end of the experiment, the mean accumulated mass ranged from a 66% increase for the largest size class to a 5800% increase for the smallest size class. • Most samples were negatively buoyant after 12 weeks of submersion. As predicted, the first samples to sink were the 0.1-mm LDPE microplastics, with 50% of samples	(Fazey & Ryan, 2016)

	Parameter	No.	Type of Study	Study approach	Limitation	Remark	Ref.
						retrieved at Week 2 already negatively buoyant	
		1.5	M	• Modeling the MP's vertical transport while considering the effect of biofilm growth, and ocean depth profiles for light, water density, temperature, salinity, and viscosity	• Chaotic behaviour was not considered. • Oscillation periods depend on several parameters, but this study just focused on the key parameters affecting particle growth and density • Wind mixing and turbulent fellow effects have not been considered	• The settling onset time is a trade-off between the particle's radius and the surface-to-volume ratio. • Larger particles have a higher collision frequency with the algae due to shear, therefore increasing their density faster compared to smaller particles. • Small particles need fewer algae to start settling, as their surface-to-volume ratio is larger. This trade-off results in the asymptotic-shaped relation between the log of the particle radius and the moment at which particles start settling for the first time	(Kooi et al., 2017)
2.	Size, Shape, Type, and Density	1.6	FW	• Investigation for the occurrence, distribution, morphology, and polymer types of MPs in the sediments • Estimating the content of MPs in the coastal sediments • Measuring the density of MPs from sediments and determining the effective	The biofouling formation in the natural environment is harder than in-situ experiment	• The density-increasing rates of MPs (PE and PP), caused by the co-contribution of biofouling and inorganic minerals, were 7.4%-12.6% and 12.1%-17.5%, respectively. • The relative abundance of MPs shapes (pellet, fiber, fragment, and foam) demonstrated that the fiber was the most abundant shape, with a mean proportion of 54.5% of the total MPs particles.	(F. Wu et al. 2020)

Parameter	No.	Type of Study	Study approach	Limitation	Remark	Ref.
			factors on the density increase of MPs in the • Site location: The Bohai Bay coast		• The small size (< 1 mm) were the most abundant type of the total MPs, with the proportions of 27.4% for < 0.5 mm and 24.6% for 0.5-1 mm, respectively, followed by 1-2 mm (18.8%), 2-3 mm (12.5%), 3-4 mm (8.8%) and 4-5 mm (8.1%). • The film-liked fragment had a higher collision frequency with the algae and other inorganic materials because of their higher surface area to volume ratio	
	1.7	FW	• Study of the density changes of plastic items (LDPE, PS, and PP) due to the fouling process • Site location: Biscayne Bay (Miami, Florida)	• The depth of the sampling was limited to 10-15 m • Samples were fixed and restricted floating exposure so they did not have the freedom of vertical movement in the water column	• The gelatinous film was apparent after a few days of exposure and was thick enough to be scraped off the surface after 1-2 weeks of exposure. • Depending on the geometry of the samples and the surface characteristics, different species of invertebrates proceeded to colonize the presumably nutrient-rich surface partially covered with algae. • The fastest rates of fouling were obtained with samples having the highest specific area	(Ye and Andrady 1991)
	1.8	FW	• Study of the process of biofouling and biodegradation of the polymers (LDPE, HDPE, and PP) • Measuring the characteristic parameters • Site location: ocean waters of the Bay of Bengal near	• The depth was limited to 3 m	• The formation of biofilms allows other macrofouling communities to deposit easily. • attachments of colonies were higher on PP, followed by that on HDPE and finally on LDPE	(Sudhakar et al. 2007)

	Parameter	No.	Type of Study	Study approach	Limitation	Remark	Ref.
				Chennai Port and Fisheries Survey of India (FSI)			
		1.9	FW	• The effects of surface characteristics of LDPE, HDPE, and PP on biofouling and the stability of these polymers exposed to seawater for one year • Site location: the ocean waters of the Bay of Bengal near the Fisheries Survey of India (FSI)	• The samples were immersed in ocean waters at a depth of 3 m • In the present study, the thickness and number of macrofoulers were not quantified	• The total suspended solids and organic matter were more on HDPE, followed by PP and LDPE	(Artham et al. 2009b)
2.	Size, Shape, Type, and Density						
		1.10	FW	• Study of the changes in MPs density and settling behaviour of three different types of MPs (PET, PP, and PVC) • Site location: Three different freshwater environments (the Niushoushan River, the Qinhuai River, and East Lake)	• Algae identified from the biofilm belong to three main phyla (blue, brown, and green algae). Of these, the green algae were below the detection and only the proportions of blue and brown algae were compared.	• The attached biofilms significantly changed the density. Density increased in high-density MPs (PET and PVC) and decreased in low-density MPs (PP). • Light MPs tend to suspend in the cages, while heavy ones are more likely to sink to the bottom, which means the formation of biofilm might be different	(Miao et al. 2021)

Parameter	No.	Type of Study	Study approach	Limitation	Remark	Ref.
	1.11	Exp	• Evaluating the effects of microparticle type (PE MPs of irregular shape, PS microspheres, and glass microspheres and particle) size on the biofilm communities that grow on MPs in wastewater and freshwater environments	• MPs biofilm in the environment would experience more complex phenomena including UV radiation, exposure, and further biofouling, which would impact particle density and community	• Microparticle type and source water were greater drivers of microbial community structures observed on microparticle surfaces than in size class.	
2. Roughness, Surface Energy, and Hydrophobicity	2.1	FW	• Evaluating the effects of surface characteristics of LDPE, HDPE, and PP on biofouling and the stability of these polymers exposed to seawater for one year • Site location: the ocean waters of the Bay of Bengal near the Fisheries Survey of India (FSI)	• The samples were immersed in ocean waters at a depth of 3 m • In the present study, the thickness and number of macrofoulers were not quantified	• There was a strong positive correlation between surface energy and total suspended solids during the first three months	(Artham et al. 2009b)

Parameter	No.	Type of Study	Study approach	Limitation	Remark	Ref.
	2.2	EXP.	• Examination of the effect of polymers (PMMA and HSR)[1] surface roughness value (SRV) on the rate of the accumulation of bacterial biofouling	• The effect of switching off the flow to remove the samples may not be the same for all samples • Repositioning the samples increases the chances that a sample slips slightly out of place and alters the local water flow. • Details about changes in fouling micro density were not recorded	• Samples of different SRVs are only readily apparent during the initial stages of fouling since by the end of the experiment there is no difference in the fouling levels on the samples, regardless of SRV. • The results produced in the laboratory are repeated in the marine environment	(Kerr and Cowling 2003)
	2.3	FW	• Study of the process of biofouling and biodegradation of the polymers (LDPE, HDPE, and PP) • Measuring the characteristic parameters • Site location: ocean waters of the Bay of Bengal near	The depth was limited to 3 m	• The contact angle of the control samples (which is a measure of the hydrophobicity) is lowest for LDPE, followed by that for HDPE and finally for PP indicating that LDPE is the least hydrophobic and PP the most hydrophobic, thus biofilm attachment was highest on PP in the first month, followed by that on HDPE and finally on LDPE	(Sudhakar et al. 2007)

[1] PMMA is a polymer derived from a single monomer and it is often used for optical ports in marine instruments. HSR is a recently developed polymer that is considerably stronger and more scratch-resistant than PMMA.

Parameter	No.	Type of Study	Study approach	Limitation	Remark	Ref.
			Chennai Port and Fisheries Survey of India (FSI)			
	2.4	FW	• Study of the changes in MPs density and settling behavior of three different types of MPs (PET, PP, and PVC) • Site location: Three different freshwater environments (the Niushoushan River, the Qinhuai River, and East Lake)	• Algae identified from the biofilm belong to three main phyla (blue, brown, and green algae). Of these, the green algae were below the detection and only the proportions of blue and brown algae were compared.	• Biofilm characteristics of PVC were more related to roughness and contact angle of MP • Hydrophobicity also affects the water absorption of algae and the degree of algae growth	(Miao et al. 2021)

1.4.1.1 Effects of MPs' physical properties on biofouling

Biofouling can increase MPs' density to the point where they sink. The amount of biofouling is affected by surface area, and MPs' size influences MPs 'surface area. A smaller MP has a greater relative surface area. Besides, buoyancy, the tendency of a body to float or to rise when submerged in a fluid, is related to item volume, so small MPs with high surface area to volume ratios, need fewer algae to start settling and start to sink sooner than large items. (Ye and Andrady, 1991.; Ryan 2015; Kooi et al. 2017).

Furthermore, biofouling permeability is another factor that impacts the MPs' sinking rate (Long et al. 2015; Xiao et al. 2012) because diatom aggregates are highly porous and the interior flow through the diatom aggregates by porous-fractal structure affects the MPs' hydrodynamic behaviour and their transport (Xiao et al. 2012). MPs, size also affects biofouling permeability, and this factor increases with size due to fractal dimension. Larger aggregates have more macropores in which water can flow through easily, thus, increasing the settling velocity of aggregates by decreasing drag forces (Long et al. 2015).

Shape, or the ratio of surface area to volume, is another effective parameter on biofouling, as elongated, flattened, or complexly shaped items have a higher surface area to volume ratio (e.g., spherical items have the lowest value) (Ryan 2015). For example, the film-like fragment has a higher surface area to volume ratio than other shapes with similar volume, Consequently, lots of organisms and matter could be absorbed onto the surface of fragments easily. However, the SA of fiber is lower than that of a film-like fragment, leading to a small number of fibers that can be settled. Meanwhile, by conducting a comparison between the fragment and fiber, it could be found that the film-like fragment has a higher collision frequency with the algae and other inorganic

materials (Kooi et al. 2017; N. Wu et al. 2020a), which could significantly increase the density of fragment.

To evaluate the effects of the MPs' type on the biofouling process, microbial studies of plastics in aquatic environments showed that the structure and composition of microbial communities vary in several polymer types, and they contain different biomass and chlorophyll-a levels (Miao et al. 2021; Oberbeckmann et al. 2014). It is demonstrated by biological and physicochemical parameters of high-density polyethylene (HDPE), low-density polyethylene (LDPE), and polypropylene (PP) that the attachments of colonies were higher on PP, followed by that on HDPE and finally on LDPE (Sudhakar et al., 2007). Thus, MPs' type is one of the key factors that influence biofilm compositions. However, it should be noted that besides this parameter, other important factors such as environmental situation may change the amount and type of microbial communities. For example, according to another field study in the same location, total suspended solids and organic matter were more abundant in HDPE, followed by PP and LDPE (Artham et al., 2009).

MPs' density is another property that can affect biofilm formation on their surfaces. An experimental study of changes in MPs densities during biofilm formation showed that the initial density of virgin MPs influences density changes by biofouling. In cases where the MPs initially have a higher density than water, biofouling further increases their density, while in cases where their density falls below 1 g/cm^3, it decreases further. This is inconsistent with previous studies' results because of some reasons. As well as bacteria, cyanobacteria and microalgae can also contribute to biofilm colonization, influencing its density. Then, the position of the MPs in the experimental devices is different because of their various densities. Thus, MP surfaces with light surfaces that tend to suspend in the cages may form biofilms differently from those with heavy

surfaces that sink. Light MP is more likely to interact with plankton, while heavy MP is more likely to interact with periphyton. This can also be a limitation in the experimental works and more in situ experiments can make up the difference between water's environmental conditions and experiments. It has a significant impact on the attachment of microorganisms and consequently the behavior of settling and floating (Miao et al., 2021).

1.4.1.2 Effects of roughness, surface energy and hydrophobicity on biofouling

The effect of roughness and surface energy on biofouling has rarely been reported for polymers. However, there are several reports on the impact of roughness and surface energy on biofouling in industrial and marine biofouling control (Allion et al., 2006) Surface roughness, measured in μm, is the most representative parameter to describe the surface texture of a material (Encinas et al., 2010).

According to experimental and fieldwork studies conducted by Kerr and Cowling (2003), the effect of surface roughness on bacterial adhesion was almost instantaneous (Kerr and Cowling 2003) while in other research, measured surface characteristics of polymers after biofouling showed a strong negative influence on fouling and a positive correlation between surface energy and total suspended solids (Artham et al., 2009b). Thus, more research should be performed to accurately recognize the biofilm formation process on MPs' surfaces based on roughness and surface energy.

The surface free energy is the available energy resulting from surface groups, molecules, or atoms that can interact with other groups, molecules, or atoms approaching the surface. Thus, with higher free energy, a surface would be more suitable for adhesion events (Callow and Fletcher 1994).

The wettability of the polymer surface is another surface characteristic that is determined by the contact angle. The contact angle indicates the hydrophobicity of the surface and has a positive

correlation with the hydrophobicity (Sudhakar et al. 2007) and hydrophobicity affects algae growth on polymer surfaces in marine environments (Miao et al., 2021a).

1.4.2 Environmental parameters

Attached biofilm growth on MPs surface in an aquatic environment is a function of environmental factors including water parameters (depth, light, temperature, dissolved oxygen (DO), and oxidation-reduction potential (ORP)), season and geographic location, nutrition levels, and types of environments (Figure 1-3). In the following sections, these parameters and their effects on MPs' biofouling are discussed and related studies are summarized in Table 1-2.

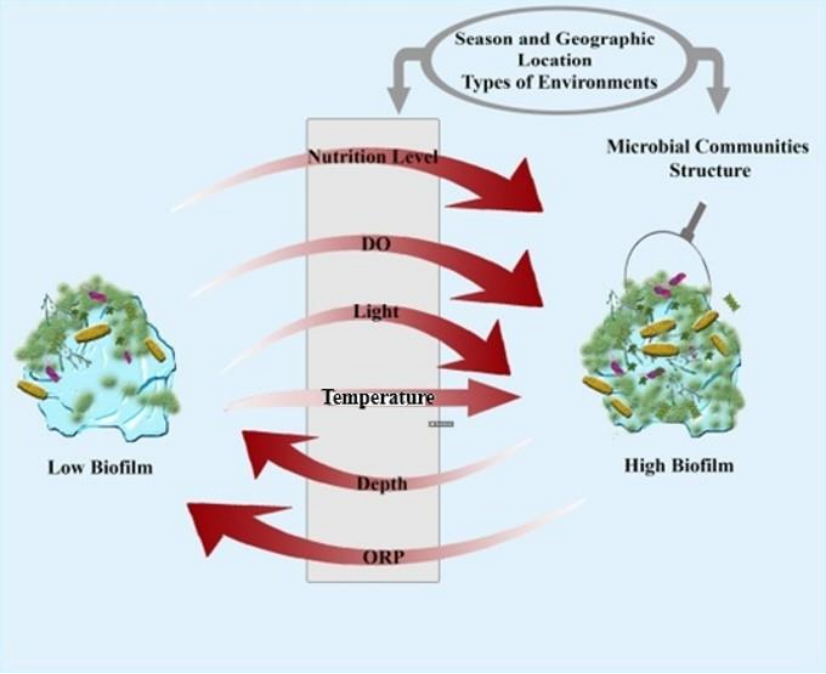


Figure 1-3 Effects of environmental parameters on the MPs' biofilm growth

Table 1-2 Effective environmental factors on the MPs' biofouling rate

Parameter	No.	Type of Study	Study approach	Limitation	Remark	Ref.
1. Water Parameters (Depth, Light, Temperature, DO, ORP)	1.1	M	See Table 1-1 No. 1.5	See Table 1-1 No. 1.5	• External forcing of light variation at the surface together with the depth profiles of light extinction, salinity, density, viscosity, and chlorophyll, in turn, influence the algae collision, growth, respiration, and settling velocity. • Some oscillations display a fixed daily period. These maximum depths are reached around noon. • Circadian cycles, or 24 h cycles, have been observed in algal behaviour, where an increase in chlorophyll-a concentrations is observed during the day, while a decrease is found during dark hours • The day: night duration affected the particle settling with increasing light duration, algae growth was enhanced, and the settling onset time decreased. • The amplitude of the settling oscillations remained similar under different light durations and algae cell volumes.	(Kooi et al., 2017)
	1.2	FW	See Table 1-2 No 1.7	See Table 1-1 No 1.7	• The density of most samples decreased markedly on being submerged because of retardation (or even reversal) of fouling brought about by the lack of sunlight in the case of algae and by predation of some macrofoulants by fish and other benthic species.	(Ye & Andrady, 1991a)
	1.3	FW	• Study of the effects of geographic location on MPs (PS and PE) biofouling in • Site location: The estuary of the Warnow river and a	• The limited depth below the low tide water level at depths	• MPs' sinking velocities decreased due to lower water temperatures and reduced light availability	(Kaiser, Kowalski, and Waniek 2017b)

Parameter	No.	Type of Study	Study approach	Limitation	Remark	Ref.
			coastal site near Heiligendamm (both in the Baltic Sea area of Germany)	between 20–100 cm.		
					• The microorganisms are more predominant in Port samples than in FSI samples. This is attributed to higher dissolved oxygen in the former site than in the latter.	
	1.4	FW	See Table 1-1 No 1.8	See Table 1-1 No 1.8	• Dissolved oxygen, biofouling, and organic matter are low at FSI but chlorophyll is higher than at Port, the latter happening because of lower oxidation and reduction potential (ORP) at the site. Water pollution leads to higher chlorophyll formation.	(Sudhakar et al., 2007 et al., 2007)
	1.5	FW	See Table 1-1 No 1.6	See Table 1-1 No 1.6	• As the increasing of water depth, the defouling activities could lead to the density decrease	(F. Wu et al. 2020)
	1.6	FW	See Table 1-1 No 1.10	See Table 1-1 No 1.10	• The biofilm characteristics of PP and PET in Niushoushan River were more related to the pH, DO and COD of the water	(Miao et al., 2021a)
2. Season and Geographic Location	2.1	FW	See Table 1-1 No 1.7	See Table 1-1 No 1.7	• Most samples (except for 22or22ofoam samples which are too low in density to sink under even very extensive fouling) foul sufficiently under Florida exposure to cause sinking at least temporarily, in a relatively short period of exposure of 7-9 weeks. This duration is likely to be geography as well as season-dependent	
	2.2	FW	See Table 1-2 No 1.8	See Table 1-2 No 1.8	• Biofouling was found to depend on the season, with loading being highest in August.	(Sudhakar et al. 2007)

Parameter	No.	Type of Study	Study approach	Limitation	Remark	Ref.
					• During the first three months, it was observed that polycarbonate which was more polar and hydrophilic than polyolefins, had higher TSS and OM, but as the seasons changed a buildup of fouling on polyolefins was observed. • On average over 12 months, polycarbonate had a higher amount of algal deposition than polyolefins. Among the three polyolefins, hydrophobicity has a role in the amount of algal deposition in the three-month sample. Apart from barnacles, other foulants like mussels, tubeworms, and hydroids were also observed. However, their relative amounts varied from one month to the other.	
	2.3	FW	See Table 1-1 No 1.9	See Table 1-1 No 1.9		(Artham et al. 2009b)
	2.4	FW	See Table 1-1 No 1.2	See Table 1-1 No 1.2	• The composition of plastisphere microbial communities in marine waters varies with season, geographical location, and plastic substrate type	(Oberbeckmann et al. 2014)
	2.5	FW	See Table 1-1 No 1.10	See Table 1-1 No 1.10	• PP squares were fully covered by biofilms in East Lake and the Qinhuai River but partially covered in the Niushoushan River. PP squares in East Lake were covered by a darker brown biofilm than those in the Qinhuai River. PET squares in East Lake were partially covered by a dark brown biofilm, while those in the Qinhuai River were fully covered, but the color was light brown. PET squares in the Niushoushan River were partially and less densely covered • The Chl-a content on each PET, PP, and PVC MP square in East Lake was higher than on those in the Niushoushan River ($p < 0.01$) and the Qinhuai River ($p < 0.01$).	(Miao et al., 2021a)

Parameter	No.	Type of Study	Study approach	Limitation	Remark	Ref.
3. Nutrition Levels	3.1	FW	See Table 1-1 No 1.6	See Table 1-1 No 1.6	MPs' density decreased due to the defouling effects induced by the limits of hydraulic and nutrient conditions in the sub-surface	(N. Wu et al. 2020)
4. Types of marine environments	4.1	FW	See Table 1-2 No 1.3	See Table 1-2 No 1.3	• Biofilm composition differed between estuarine and coastal stations • Sinking velocities of PS increased by 16% in estuarine water (salinity 9.8) and 81% in marine water (salinity 36) after 6 weeks of incubation • Biofouling did not cause PE to sink during the 14 weeks of incubation in estuarine water, but PE started to sink after six weeks in coastal water when sufficiently colonized by blue mussels Mytilus edulis	(Kaiser et al. 2017)
	4.2	Review	• Comparing the marine plastisphere assemblages with freshwater habitats • Study of the behaviour and ecological impacts of MPs pollutants by analyzing the key differences and commonalities between environments	• The effects of movement between aquatic and terrestrial habitats on plastic-associated biofilms have not been studied	• Freshwater and marine environments are different in environmental and hydraulic parameters which are likely to affect biofilm formation on MPs' surface • Taxonomy associated with MPs has mostly been studied in marine ecosystems, there remains a particular lack of information concerning plastisphere assemblages within freshwater	(Harrison et al. 2018)

1.4.2.1 Effects of water parameters (depth, light, temperature, DO, ORP) on biofouling

Depth, light, and temperature are interdependent water parameters that have important effects on MPs' biofouling.

In the natural environment, the light intensity is not uniformly at the optimum value for the maximum growth rate. It varies as a function of depth because of the natural turbidity and as a function of the daytime. Thus, the algae in the lower layers are exposed to intensities below the optimum and those at the surface may be exposed to intensities above the optimum so that their growth rate would be inhibited (Di Toro et al., 1971). The studies about the spontaneous effects of light and temperature on the growth rate of algae show that the optimum light intensity also depends on temperature (Sorokin & Krauss, 1962; Tamiya et al., 1953). Algal growth rate increases with temperature up to an optimal and decreases with further increases (James & Boriah, 2010). Hence, the water temperature over depth should be considered as another key factor that influences biofouling and MPs' sinking velocity (Kooi et al., 2017).

Algae growth rate reduces in non-optimum light intensity; hence, the growth rate may reduce at any light intensity. The light is attenuated concerning depth which is defined by the extinction coefficient. Thus, the reduction of the saturated growth rate resulting from the non-optimum light intensity may occur at any depth (Di Toro et al., 1971).

Dissolved oxygen in the marine environment is another important parameter to biofilm formation on the MPs' surface. Microorganisms are more predominant in the environment with higher dissolved oxygen.

Oxidation and reduction potential are an indication of the effect of aeration on the water. The lower ORP leads to higher chlorophyll formation (Sudhakar et al., 2007).

1.4.2.2 Effects of season and geographic location on biofouling

Microbial communities of plastic vary in structure and composition about geographical location and season, but those similarities also exist between these plastic communities (Oberbeckmann et al. 2014).

Geographic and seasonal differences in the structure and composition of biofilm on MPs' surface in marine environments have been considered in several studies (Oberbeckmann, Osborn, and Duhaime 2016), however, further investigation is needed in freshwater (Harrison et al. 2018).

1.4.2.3 Effects of nutrition levels on biofouling

Nutrient availability is another important water biochemical condition regulating the biofouling process. Under light-saturating conditions, microalgae growth depends on the availability of nutrients such as nitrogen, phosphor, carbon, and ammonia. Furthermore, algae growth rate can also be reduced by the limitation of carbon dioxide because it is used as a carbon source during photosynthesis. Silicate is another nutrient that affects the growth rate of diatoms (Di Toro et al., 1971; Lee et al., 2015).

1.4.2.4 Effects of type of environments on biofouling

As mentioned, ambient conditions are likely to influence biofilm formation on the surface of MPs. Many of these conditions vary between freshwater and marine habitats (e.g., low temperature and less light available in deep water compared to shadow habitats). In terms of the nutrient value, in contrast to nutrient-poor conditions present within the open ocean, inland and coastal waters receive high fluxes of nutrients from the surrounding environment including organic matter input and upwelling, high concentrations of nutrients which are released by agriculture and other human activities. Microbial colonization and the subsequent biofilm formation on plastics are affected by the type of environment. Most freshwater environments are eutrophic, which means that MPs may

form biofilms at a faster rate and have a richer microbial community in freshwater environments (Harrison et al. 2018; He et al. 2022; Ramsperger et al. 2020).

Furthermore, continuous downstream movement of water and flow rate are the key distinction between freshwater and marine ecosystems that is likely to affect plastic-associated biofilm composition and activity, due to accompanying shifts in the surrounding environmental conditions (Harrison et al. 2018). The microscopic biofilm of the particles also differs greatly between estuarine and coastal waters (Kaiser, Kowalski, and Waniek 2017b).

1.5 Biofouling effects on MPs' vertical transportation and reviewed studies

MPs can be positively and negatively buoyant. In contrast to buoyant particles, MPs that are denser than water (negatively buoyant), start to sink when they enter a water body. The density of the buoyant MPs increased by biofouling until the particles started to settle. After initial settling, MPs keep moving up and down in the water column due to the dynamics of the differences between the density of the seawater and the plastic particle. One of the main reasons for these density differences is the biofilm volume (number of algae) on the MP's surface which changes by the fouling and defouling process (Kooi et al. 2017). The interaction of these different growth and loss processes leads to moving MPs up and down as shown in Figure 1-4.

MPs' dynamic density, as well as their shape and size, are from main reasons which make MPs' transport different from other sediments such as sand and silt in aquatic environments. These characteristics can change with time by biofilm formation on the MPs surfaces(Chubarenko et al. 2016; H. Zhang 2017). Thus, MPs transport in aquatic environments deviates from what traditionally occurs for other particles (Khatmullina and Isachenko 2017).

Hence, due to the important role of biofouling in the MPs characteristics changes and its effects on their specific behaviour, it has been the subject of various recent studies.

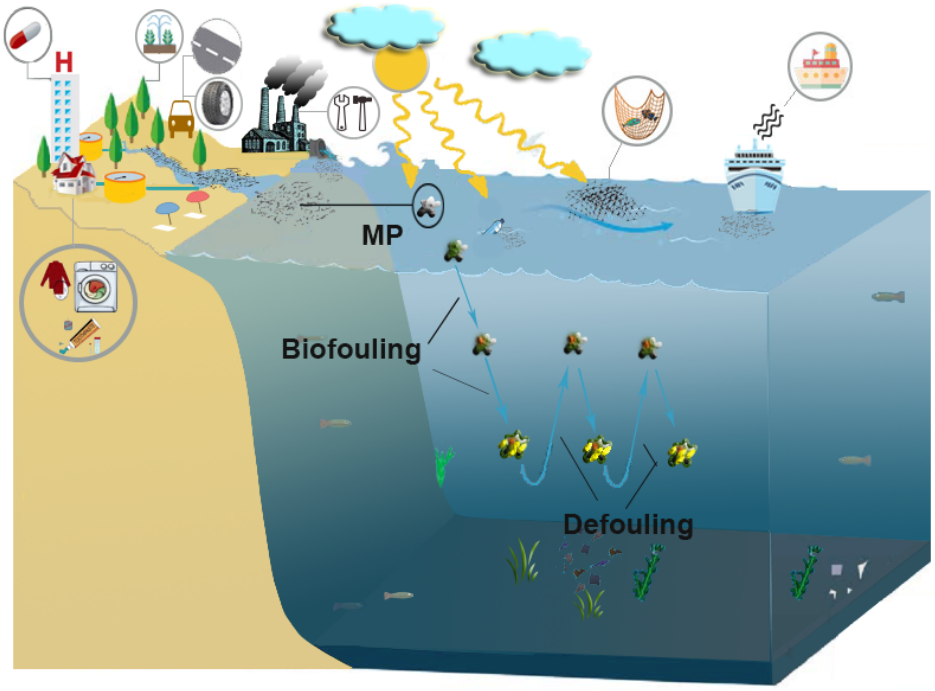


Figure 1-4 MPs enter the aquatic system from different sources and then keep moving up and down by biofouling and defouling process

1.6 Wastewater effects on biofouling

Microplastics have become a global environmental concern due to their widespread presence in aquatic systems. The release of wastewater effluent into freshwater systems can influence various ecological processes, including biofilm formation on microplastics that already persist in these environments from other sources (Herzke et al. 2021). When wastewater effluent, which typically contains a blend of residual organic compounds, nutrients, and diverse microorganisms, is introduced into freshwater ecosystems, it can alter the microbial dynamics and nutrient balance. This introduction can have significant consequences for the biofilm formation on surfaces, including the surfaces of microplastics that have originated from other sources(Wakelin, Colloff, and Kookana 2008).

Microplastics, by their nature, offer an ample surface area for microbial colonization. The presence of increased nutrients and organic matter from the effluent can accelerate biofilm development on these microplastic surfaces. For instance, effluent rich in phosphorus and nitrogen can promote the growth of certain algae and bacteria. As they proliferate, these microorganisms can create dense biofilms on the microplastics, potentially leading to shifts in microbial community compositions within freshwater systems (Chen et al. 2020).

Moreover, biofilms can modify the properties of microplastics. The accumulation of microbial layers can affect the plastic's buoyancy, potentially leading to increasing sedimentation or altered distribution patterns within the freshwater environment. This change can influence where and how microplastics interact with other aquatic life, from small invertebrates to larger fish, altering ingestion patterns and potential exposure to any associated pollutants (Gaylarde et al. 2023; Semcesen and Wells 2021).

Certain compounds in the wastewater effluent might promote the growth of specific pathogenic or antibiotic-resistant bacteria. If these bacteria favor the microplastic surfaces for colonization, there is a risk of these plastics becoming vectors for such organisms, further complicating the ecological impacts (Pham, Clark, and Li 2021).

In essence, while the microplastics in freshwater might originate from various sources, the introduction of wastewater effluent can significantly influence the biofilm formation on their surfaces. This relationship underscores the intricate interactions between anthropogenic pollutants and ecological processes in freshwater systems.

Lake Ontario, as one of North America's significant freshwater bodies, is no exception. The lake's proximity to urban areas makes it susceptible to receiving MPs and other contaminants, especially from wastewater outlets (Ballent et al. 2016; Grbić et al. 2020). These outlets are pivotal points of

interest as they may directly introduce both microplastics and a suite of associated contaminants, potentially influencing the biofouling process.

Biofouling, the accumulation of microorganisms, plants, algae, and even small animals on submerged surfaces, can significantly modify the physical and chemical properties of the fouled materials even their interaction with aquatic life (Lobelle et al., 2021). It's very important to understand how biofouling happens near wastewater outlets because MPs might carry pathogens and other pollutants.

1.7 Research gaps

The evaluation of the effects of releasing wastewater effluent into freshwater on microplastics biofouling is an evolving field of study, and several research gaps persist. Identifying these gaps can pave the way for a more comprehensive understanding of how wastewater effluent affects biofilm formation. One of these research gaps is initial information on MP biofouling that includes data representing the initial conditions before starting any experiment. This initial data is crucial because it allows for an understanding of the situation before any changes or interventions are applied. This data regarding the extent and nature of biofouling on microplastics in freshwater systems without wastewater influences is lacking. Such data is crucial to discern the specific impacts of wastewater effluents.

Another important research gap is related to the specificity of microorganisms. The role of specific microorganisms present in wastewater effluent that may preferentially colonize microplastics remains inadequately explored.

The experimental method proposed in this study offers the opportunity to assess the influence of wastewater effluent and delineate the effects of various microorganisms and their combinations on the biofouling of microplastics.Another research gap observed in on-site studies is the lack of long-

term investigations. Long-term studies present challenges, particularly in rapidly changing environmental conditions. However, this experimental setup offers the opportunity to examine various environmental and hydraulic parameters under controlled laboratory conditions.

1.8 Research motivation

Because of wastewater effects on biofouling, this experimental work aims to conduct procedures for evaluating the biofouling process of MPs in Lake Ontario in conditions near wastewater outlets. The study aims to understand how bacteria, water parameters, and flow rate impact the biofilm formation on microplastics.

Addressing the research gaps mentioned in section 1.7 will provide a more holistic understanding of the effects of wastewater effluent on microplastics biofouling in freshwater systems and will aid in formulating better management and mitigation strategies. Evaluating and controlling all these effective parameters in laboratory scales is easier and more applicable. Besides, MP's in-situ biofouling studies without controlled environmental parameters are a time-consuming process. Hence, with the lab scale study pilot presented in this study evaluating the above effective parameters is more applicable with less time.

Furthermore, to statistically evaluate recent studies on MPs biofouling in aquatic environments, the Web of Science database was used to retrieve the relevant publications from 2015 till Oct. 2023.

About 43% of papers were relevant to biofouling environmental studies. 43% of studies on the MPs biofouling were in the field of MPs hydrodynamics and about 14% of the studies focused on biofouling microbiological studies.

In these studies, only 18% were freshwater studies, and the rest were in other aquatic environments. Although the amount of research in MP biofouling in freshwater has increased since 2020, there

is still a gap in this type of environment and more research on the freshwater system is needed. Additionally, 25% of all studies were experimental, while others involved fieldwork or modeling. We therefore decided to design a lab pilot experiment and to focus on freshwater as the ambient water in the present study due to the lack of studies that are experimentally conducted in freshwater.

Chapter Two: Methodology

In this study MPs' biofouling process in Lake Ontario, in conditions near wastewater outlets, was evaluated (Figure 2-1). Bacteria isolated from wastewater effluent were grown in standard synthetic freshwater. The protocol including chemicals and their concentrations to prepare synthetic freshwater was selected based on Lake Ontario's water alkalinity. After evaluating the effects of carbon and nitrogen sources on bacteria growth in this ambient water, it was determined which of these isolated bacteria could grow in this synthetic water. These types and their different combinations were then studied on two different sizes of polyethylene microplastics in different mixing velocities to determine the effects they had on bacterial growth and biofilm formation. Bacterial growth was assessed by measuring the optical density of the culture and glucose consumption using the dinitrosalicylic acid test.

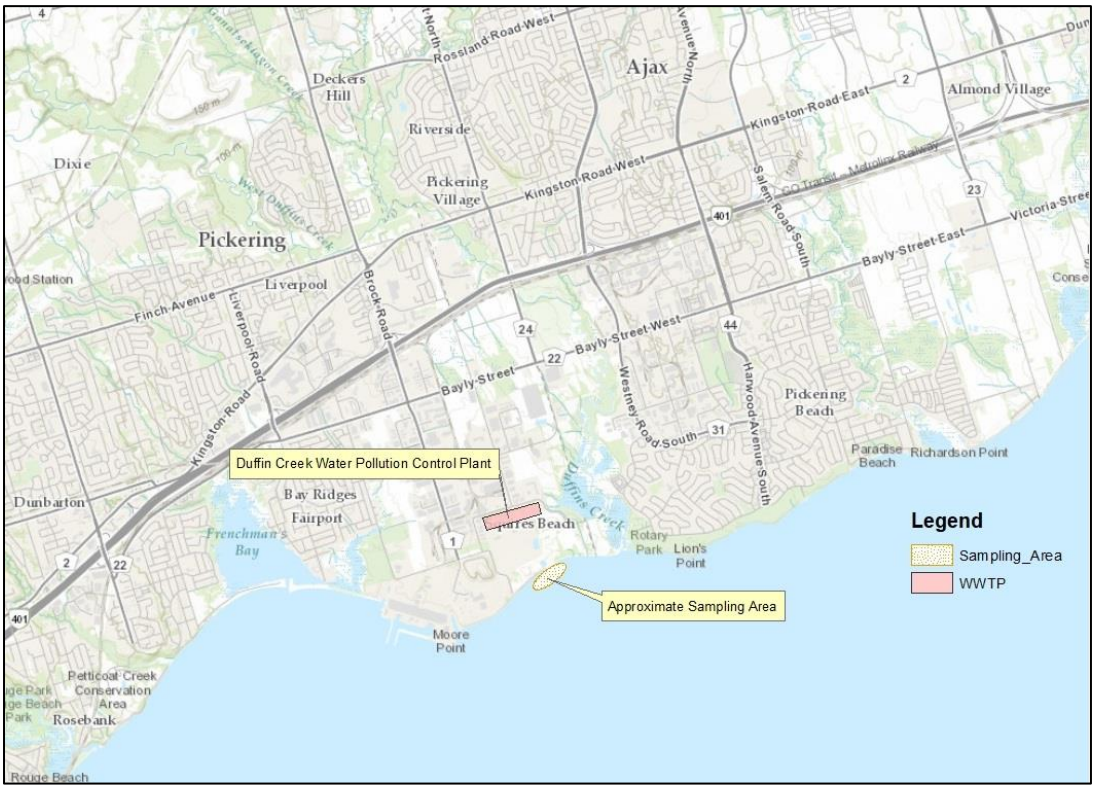


Figure 2-1 Wastewater treatment plant location and approximate sampling area

(Helle et al., 2000). Continuous systems were chosen to examine how varying flow rates affect the biofouling rate. Hydrodynamic conditions control two interdependent parameters, that are mass transfer and shear stress, which will, in turn, significantly influence many of the processes involved in biofilm development. By employing continuous systems, a constant flow of fresh medium, nutrients, and microplastics could be maintained, ensuring a consistent growth environment for the bacteria throughout the experiment. Increased shear tends to increase the detachment rate by the physical removal of individual cells (erosion) or larger pieces of biofilm (sloughing), while increased mixing tends to increase the growth rate by reducing transport limitations and increasing the nutrient supply (De Beer and Stoodley 2006).

The use of continuous systems allows for real-time data collection and monitoring, which is crucial when investigating dynamic processes such as biofouling. The selection of continuous systems as the experimental approach was driven by the need for a controlled environment to explore the impact of flow rates on biofouling, with a focus on continuous data collection and real-time monitoring, all of which are essential for comprehending the dynamic nature of the biofouling process (Flemming 2003).

In order to assess the influence of flow rate, three continuous systems were established, each featuring distinct flow rates, and optical density (OD) and dissolved organic carbon (DNS) were measured daily. This approach was implemented to ensure the maintenance of an ideal growth environment for the bacteria during the entire experiment.

The functionality of the systems was periodically verified by checking levels of chemical oxygen demand (COD), ammonia, and phosphorus. Additionally, protein concentrations were measured in the water to assess and characterize biofilm formation.

Microscopic studies on the surface of MPs were observed to examine the presence of biofilm formation on the surface of microplastics.

2.1 Materials

2.1.1 Chemicals and reagents

The different chemicals and materials used in the current investigation are listed below:

2.1.1.1 Synthetic water preparation

The components of the synthetic water were deionized water, $NaHCO_3$, $CaSO_4$,$2H_2O$, $MgSO_4$, and KCl. All chemicals used for synthetic water preparation were of analytical grade and purchased from Sigma Aldrich.

2.1.1.2 Nutrients and natural organic matter

Nutrients including Glucose ($C_6H_{12}O_6$), H_4ClN (ammonium chloride), $H_9N_2O_4P$ (diammonium phosphate (DAP)) were purchased from Sigma Aldrich. Suwannee River NOM (Natural Organic Matter) was also added to synthetic water that was provided by the International Humic Substances Society (IHSS).

2.1.1.3 Media preparation

Nutrient broth and agar were bought from TekniScience Inc. to prepare media for bacterial growth.

2.1.1.4 DNA test

To identify the types of bacteria growing in ambient water, DNA was isolated using Quick-DNA Miniprep Plus Kit (D4068S) from Zymo Research.

2.1.1.5 Dinitro salicylic acid (DNS) test

The analytical grade chemicals required for the DNS test including 3,5 Dinitro salicylic acid (DNS) reagent, NaOH, and Sodium Potassium Tartrate were purchased from Sigma Aldrich.

2.1.1.6 COD, Nitrogen- ammonia test, and total phosphorous test

COD Digestion Vials, High Range, AmVer™ Salicylate Test 'N Tube™ High Range Ammonia Nitrogen (Range: 0.4 – 50.0 mg/L NH_3-N), and phosphorus (Reactive) TNTplus Vial Test (5-90 mg/L PO_4) were purchased from Thermo Fisher Scientific.

2.1.1.7 Protein content measurement

A modified Lowry Protein Assay Kit., to measure protein content was obtained from Thermo Fisher Scientific.

2.2 Methods

2.2.1 Synthetic water preparation and adding nutrients

Early studies including (Reinwand 1969) have shown that the average total alkalinity of Lake Ontario is 101.7 ppm $CaCO_3$. Thus, it is categorized as hard water. Accordingly, synthetic hard freshwater was prepared (Figure 2-2).

To prepare 1 liter of synthetic, hard, reconstituted water following the USEPA 2002 protocol, It was started by placing 900 mL of deionized water in a cleaned plastic carboy. 120 mg of $MgSO_4$, 192 mg of $NaHCO_3$, and 80 mg of KCl to the carboy, and it aerated overnight. In a separate flask, 120 mg of $CaSO_4.2H_2O$ was dissolved in 100 mL of deionized water, then it was combined with the 900 mL solution in the carboy, ensuring thorough mixing. This process yields 1 liter of the desired synthetic, hard, reconstituted water.

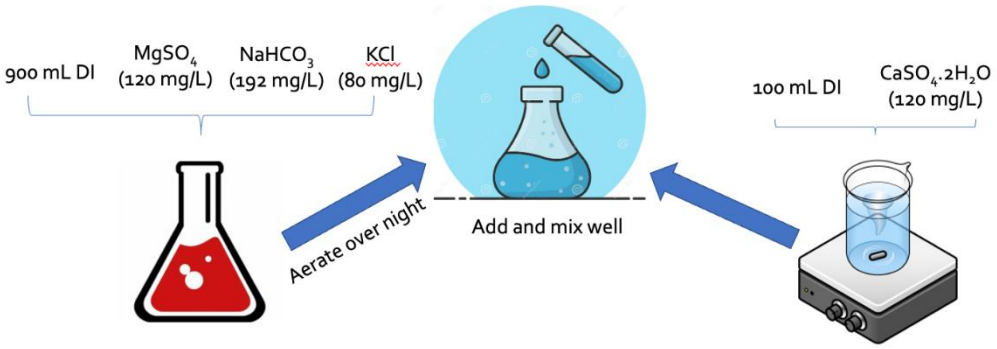


Figure 2-2 Preparing 1L synthetic water based on USEPA 2002 method

To promote bacterial growth and provide microorganisms with the required nutrients, certain compounds were added to ambient water. Glucose ($C_6H1_2O_6$) with a concentration of 2g/L was added as the carbon source (Sturm, Schuhen, and Horn 2022). As a nitrogen source, ammonium chloride (H_4ClN) was included, supplying readily available ammonium ions (NH^{4+}). Diammonium phosphate ($H_9N_2O_4P$) was added as a phosphorus source, providing accessible phosphate ions ($PO^{4^{3-}}$) for microbial growth (Khan et al. 2016). To ensure an optimal balance between growth and inhibit excessive EPS production, the carbon-nitrogen-phosphorus (C/N/P) ratio was maintained at 100/10/1 (Rahman 2013; Lechevallier, Schulz,' And, and Lee 1991). Additionally, a small concentration of Suwannee River NOM, derived from the Suwannee River, was included to introduce complexity with its diverse mixture of organic compounds (Liu et al. 2003). This setup promotes microbial growth and creates an ideal environment for conducting research.

2.2.2 Isolating bacteria from effluent

Wastewater treatment plant effluent was collected from Keswick Water Resource Recovery Facility (WRRF), Ontario, Canada in June 2021. Bacteria were isolated using the serial dilution method.

Bacterial isolation involves separating a single bacterial species from a mixed culture using techniques like pouring, spreading, streaking, and serial dilution. It allows for the observation of bacterial growth on solid or liquid media, including automated systems that detect bacteria through indicators like carbon dioxide production. This method is essential for examining the characteristics and behavior of specific bacterial (Figure 2-3).

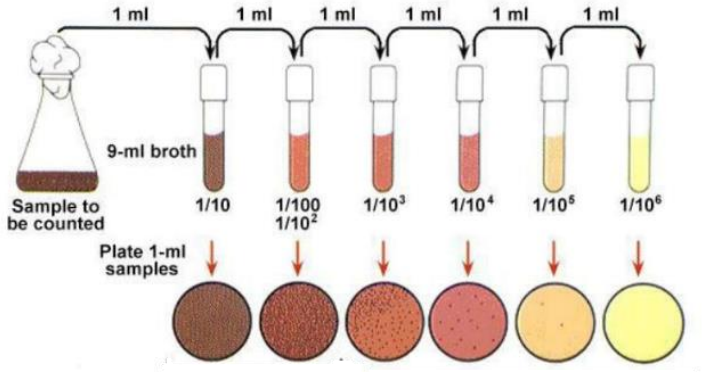


Figure 2-3 Representation of serial dilution method for bacterial isolation

The serial dilution method is the cornerstone technique in microbiology, especially when the objective is to isolate and enumerate bacteria from a mixed or dense sample. In essence, the method involves the systematic dilution of a sample to reduce the bacterial concentration to a level where individual bacteria or groups can be effectively isolated and grown as distinct colonies on a solid medium (Dunham 2010).

To begin, a 1 mL sample containing bacteria is mixed with 9 mL of a sterile solution, such as saline or a buffer solution. This effectively reduces the concentration of the bacteria. This diluted sample is then used as the starting point for further dilutions, where a small volume is taken and mixed again with the sterile solution. This step-by-step dilution process was repeated seven to ten times for different bacteria, to create a series of tubes with decreasing concentrations of bacteria.

Once the dilutions are prepared, they're plated onto solid growth media, typically agar plates. By spreading a small volume of each diluted sample onto these plates, the dilution has been sufficient to allow individual bacteria to grow spaced apart from each other. After incubating these plates for a period, individual bacterial colonies can be observed and studied (Cole and Rankin 2021).

This method was applied in this study to separate and isolate bacteria from wastewater effluent. The procedure involves preparing a series of test tubes containing a liquid medium, and then, transferring small volumes of the original bacterial suspension into successive test tubes to create a dilution series. Each transfer represents a specific dilution, such as 1:10 or 1:100. The contents of each test tube are thoroughly mixed to ensure proper dilution. After the desired dilutions are prepared, a small volume from the last test tube is plated onto solid nutrient agar or a suitable medium. Following incubation, individual bacterial colonies arise on the agar plates, originating from single bacterial cells present in the diluted suspension. These colonies can be selected and isolated based on distinct characteristics through streaking onto fresh agar plates, allowing for the obtainment of pure cultures for further study of bacterial properties, such as morphology, physiology, and pathogenicity. Serial dilution is an efficient and controlled way of studying the characteristics of isolated bacteria.

2.2.3 Growing bacteria in the nutrient broth (NB) and plate

To study bacteria and other microorganisms, it is necessary to grow them in controlled conditions in the laboratory. Growth media contain a variety of nutrients necessary to sustain the growth of microorganisms. There are two commonly used physical forms of growth media: liquid media and solid growth media.

Nutrient broth is typically made of a powdered beef extract that contains peptones (simple proteins). CRITERION™ Nutrient Broth was used in this experiment. The formulation of

CRITERION™ Nutrient Broth is composed of peptone and beef extract. Nutrients necessary for the replication and growth of a large number of non fastidious microorganisms are provided by the simple formulation. Water-soluble substances including carbohydrates, vitamins, organic nitrogen compounds and salts are present in beef extract (Pelczar, 1986).The powder is dissolved in water, put in test tubes, and sterilized.

To grow bacteria in a liquid broth, first, nutrient broth was prepared at a concentration of 9g/L and sterilized using autoclaving. Then, 100 µL of bacteria was added to each vial containing 10 mL of the nutrient broth. The vials were placed in a shaking incubator set at 200 rpm and 30°C which is a common temperature for growing many types of bacteria, After a designated incubation period, the vials were centrifuged to separate the bacterial cells from the liquid medium. The optical density (OD) of the bacterial suspension was measured, which serves as an indicator of bacterial growth. To ensure consistency, an equivalent volume was calculated and added to each vial to achieve an OD of 1, and then was added to 10 mL of nutrient broth. The OD was then measured at regular intervals, typically every 2 hours, to determine the growth kinetics of the bacteria over time. This procedure allows for the monitoring and analysis of bacterial growth patterns and kinetics in a controlled liquid environment.

Six unknown types of isolated bacteria that have been grown in the NB were added to synthetic freshwater to determine the types of bacteria that can grow in our ambient water.

To grow bacteria on a plate, first NB was prepared at a concentration of 9 g/L and agar was added at a concentration of 1.5 g/100 mL. The mixture was then autoclaved to ensure sterility. Next, 100 µL of bacteria was added to each vial containing 10 mL of the nutrient broth with agar. To prevent contamination, a sterile metal loop was used to collect bacterial samples. The loop was gently swiped over a surface, such as a swab, to collect bacteria, and then the loop was streaked in a

zigzag pattern across the surface of the agar. The plates were then placed in a 30°C incubator and allowed to incubate for at least 24 hours. This procedure provides a solid growth medium for bacteria and allows for the observation and analysis of bacterial colonies that develop on the agar plate over time.

2.2.4 Bacteria in ambient water

Bacteria that had previously demonstrated growth in synthetic freshwater and their several combinations were added to the ambient water. Then, Species that showed the most growth using these added nutrients were selected for further study.

In a batch system that included two different sizes of MPs (355-425 µm and large 1-2mm), the effects of growing these three types and their different combinations including T1 (WW1), T2(WW2), T3 (WW5), T4 (WW1+WW2+WW5), T5 (WW1+WW2), T6 (WW1+WW5), and T7 (WW2+WW5) were studied. These two parameters and the effect of change in shaking speed during the growth process, have been studied. Ambient water was tested for bacterial growth. Carbon and nitrogen sources were manually added to each sample.

2.2.5 Deoxyribonucleic acid (DNA) test to identify the type of bacteria

Quick-DNA™ Miniprep Plus Kit – Zymo Research was used in this experiment to identify the types of bacteria involved in several steps (Figure 2-4). Initially, up to 200 µl of the bacterial sample was added to a microcentrifuge tube, followed by the addition of BioFluid & Cell Buffer (Red) and Proteinase K. After thorough mixing and incubation at 55˚C for 10 minutes, the digested sample was mixed with Genomic Binding Buffer and transferred to a Zymo-Spin™ IIC-XLR Column for centrifugation. The flow-through was discarded, and subsequent wash steps were performed using DNA Pre-Wash Buffer and g-DNA Wash Buffer. The DNA-bound column was then eluted with DNA Elution Buffer or water. The eluted DNA could be used immediately for

molecular-based applications or stored at ≤ -20ºC for future use. This protocol aimed to extract high-quality DNA from the bacterial samples, and identification of bacterial types.

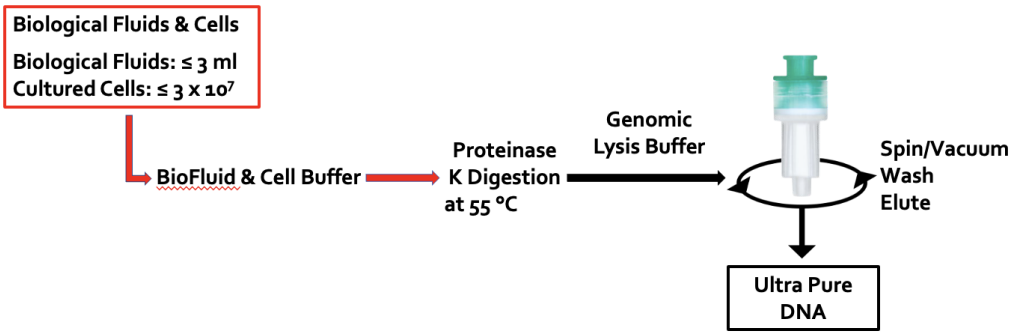


Figure 2-4 DNA isolation

This eluted DNA was used for DNA sequencing at McMaster University with ABI BigDye v1.1. The standard sequencing conditions were: annealing at 50 °C, extension at 60 °C, 2.5 mM MgCl2, and 0.2 mM primer. After sequencing, we removed extra dye using magnetic beads and put the clean reaction in the 3730 DNA automated sequencer.

The 3730 DNA Analyzer, a fully automated, multi-color DNA system, was used. It goes from loading samples to analyzing data by itself. Typically, if the DNA and primer are good and in the right amounts, and the machine works well, we get 700-900 base pairs from a standard reaction. Here's how the machine works: It puts the DNA sample into a capillary using electricity. The DNA, being negatively charged, moves into the capillary. This process is called electrokinetic injection. Sometimes, unwanted substances also get into the capillary, which can cause problems. Clean DNA is crucial for a successful reaction. Next, the capillary goes into a buffer, and with electrical current, the DNA moves towards a window where it's detected. The machine measures the fluorescence of each DNA base and identifies them (Weissman, 2011).

2.2.6 Setting-up the continuous system

The evaluation of biofouling formation on MPs due to changes in flow rate is essential to determine the impact of flow dynamics on the formation and buildup of unwanted biological materials on surfaces. Biofouling can significantly affect the efficiency and functionality of various systems, especially those related to fluid flow and water treatment. Thus, understanding the nuances related to flow rate is of paramount importance.

2.2.6.1 Setup description

To ensure a comprehensive and parallel assessment, three distinct systems started to work simultaneously, with the same environmental parameters but with different flow rates. Each of these systems comprises the following components:

Adjustable flow rate peristaltic pump: As the name suggests, this device enables the setting and adjustment of flow rates. A peristaltic pump functions by the sequential compression and relaxation of a flexible tube, ensuring a consistent and controllable flow of the fluid.

Three-liter Beaker with an Aquarium Aerator Pump: This setup serves a dual purpose. First, the beaker acts as the reservoir or source of the fluid. Second, the aerator pump ensures that the fluid is sufficiently oxygenated, mimicking a real-world aquatic system and ensuring the viability of any microorganisms involved in the biofouling process.

250 mL Glass Buchner Filtering Funnel: This device, equipped with a coarse filter of 80-120 um granularity, serves the primary function of filtration. With its specific dimensions (76 mm inner diameter and 80 mm depth), it is designed to hold and filter the fluids passed through it. Figure 2-5 shows the schematic design of a continuous system.

The continuous system was equipped with a camera to monitor the water elevation to prevent overflow during the working period. This camera's primary function was to observe the water level or elevation. Monitoring this ensured that there was no unintended overflow, which might

otherwise disrupt the experiment or cause potential hazards. Figure 2-6 illustrates the camera's positioning and the system's water level monitoring mechanism.

Before the initiation of the experiment, all systems and sampling tools were sterilized using an autoclave. The process includes the sterilization of the tools and samples at 121 °C and 15 psi pressure for 30 minutes. This step is crucial, as the presence of any external contaminants could compromise the integrity of the results.

This setup allows researchers to make definitive conclusions about the role of flow rate in MP's biofouling. By maintaining controlled variables and ensuring the sterility of the environment, the experiment's results are set to be both accurate and replicable.

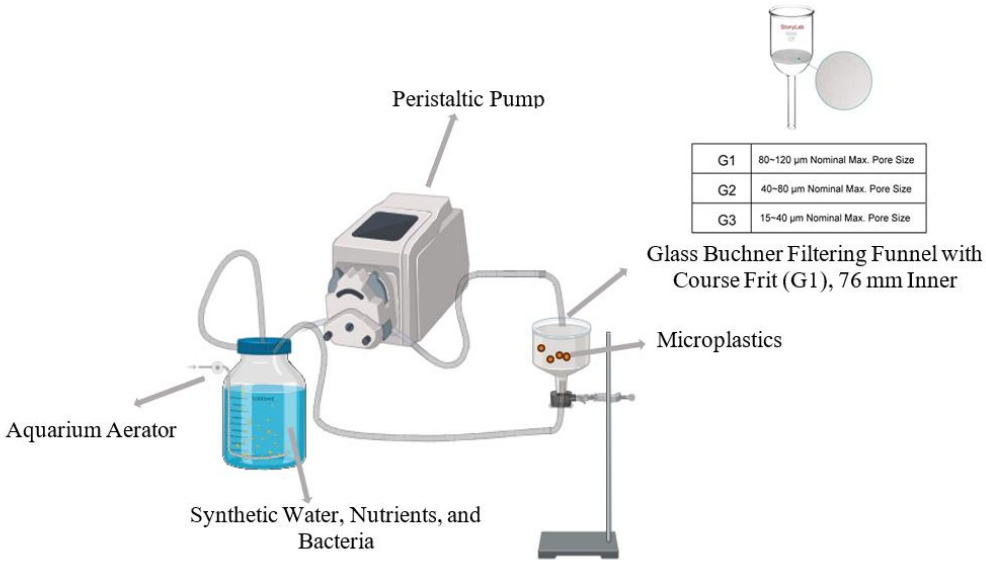


Figure 2-5 Schematic design of a continuous system

Figure 2-6 Setting up the continuous systems

2.2.7 Optical density test

To monitor the concentration of pure cultures, the light scattering techniques have the advantage of being rapid and non-destructive. However, this method measures neither cell numbers nor CFU (colony-forming unit). Light scattering is most closely related to the dry weight of the cells. The light passing through a suspension of microorganisms is scattered, and the amount of scattering is an indication of the biomass presence in the suspension. In visible light, this appears “milky” or “cloudy” to the naked eye. It follows from this that if the concentration of scattering particles becomes high, then multiple scattering events become possible. The optical density increases as the bacterial population grows, due to the increased light scattering by the cells. This provides a good quantitative estimate of the bacterial population, useful for comparing growth under different conditions. However, it's important to note that OD is not an absolute measure of cell count; it cannot distinguish between live and dead cells and may not detect very low cell concentrations. Additionally, OD readings can be influenced by other particles in the culture medium that absorb or scatter light, potentially affecting accuracy. Despite these limitations, OD remains a widely used

method in both research and industrial settings, valued for its simplicity, speed, and reproducibility (Beal et. al., 2020).

Due to the above-mentioned advantages, in the current study, optical density was measured using a “Hach DR 6000 UV VIS” spectrophotometer at the wavelength of 600 nm. In general, we followed the instruction provided by Koch (1994). In summary, the process involves preparing the sample, setting the appropriate wavelength on the UV-VIS spectrophotometer, calibrating the instrument using a blank or reference solution, measuring the absorbance (or optical density) of the sample, and then interpreting the results in the context of the specific experiment or application (Koch, 1994).

It is important to have the cells in the known physiological state of growth. As the cell size varies with the phase of growth (lag, log, and stationary phases) the approximate relationship between absorbance and CFU will also vary. A recommended practice is to pass a single well-isolated colony twice on overnight cultures surface streaks from the refrigerated stock, harvesting the rapidly growing culture from the second passage for the preparation of vegetative cells (Gilbert et al., 1987).

A second source of concern might be the cuvette used for the measurement – care must be taken to maintain the correct orientation of the cuvette, and to protect it from damage that could affect the passage of light. Finally, it is necessary to blank the spectrophotometer (adjust the absorbance reading to zero) using a standard, either water or the suspending fluid, and maintain this practice (Sutton, 2006).

2.2.8 Dinitrosalicylic acid (DNS) test

In the method used to measure the concentration of reducing sugars released during certain biochemical processes, such as the breakdown of carbohydrates by bacteria or other

microorganisms. This test is based on the principle that reducing sugars under acidic conditions will reduce the 3,5-dinitrosalicylic acid to 3-amino,5-nitrosalicylic acid, which produces a reddish-brown color. The intensity of this color is directly proportional to the concentration of reducing sugars present, and it can be measured using spectrophotometry. In the context of studying bacterial growth, the DNS test can indirectly indicate bacterial activity, particularly for bacteria that metabolize carbohydrates. When bacteria break down carbohydrates, they release reducing sugars into the surrounding environment. By measuring the concentration of these sugars using the DNS test, one can infer the extent of carbohydrate metabolism, which is a component of bacterial growth and activity (Reese et. al., 1950).

The following protocol was followed, as described by Soozanipour and Taheri-Kafrani (2018). Firstly, a xylan solution with a concentration of 1% (w/v) was prepared by dissolving beechwood xylan in 20 mM phosphate buffer at pH 6.5. This solution served as the substrate for the enzymatic reaction. The reaction mixture, consisting of the substrate and either immobilized xylanase or free xylanase, was then incubated at 60°C for 15 minutes under shaking conditions. Following incubation, the immobilized xylanase was separated from the solution using an external magnetic field. Next, 2 mL of 3,5-dinitrosalicylic acid reagent was added to the separated supernatant, and the reaction was terminated by heating it at 100°C for 5 minutes. At the end of the process, the absorbance of the released reducing sugars in the reaction was measured at 530 nm and enzyme activity was calculated using a standard curve (Soozanipour & Taheri-Kafrani, 2018). This protocol allowed for the quantification of reducing sugars produced by xylanase activity, providing insights into the efficiency of the enzyme and its potential applications.

To prepare 500 mL of DNS reagent, the following procedure was used. A 1-liter glass bottle was covered with aluminium foil and agitation was maintained with a magnetic stirrer throughout the

preparation. First, 5 g of DNS reagent was added to the bottle, followed by the addition of 50 mL of distilled water. The mixture was allowed to sit until most of the DNS reagent was dissolved. Separately, 8 g of NaOH was dissolved in 100 mL of water, and this NaOH solution was slowly added to the bottle. Then, 150 mL of distilled water was added. Sodium potassium tartrate was added in batches of 30 g, ensuring each batch was dissolved before adding the next one. If the sodium-potassium tartrate takes time to dissolve, gentle heating can be applied up to a maximum of 35 degrees Celsius. Once all sodium potassium tartrate was dissolved, 100 mL of distilled water was added, and the solution was shaken for approximately 30-60 minutes without heating. Afterwards, the DNS solution was poured into a measuring cylinder and distilled water was added to reach a final volume of 500 mL. The prepared DNS reagent was stored in an amber container for future use.

2.2.9 Evaluating the function of systems

2.2.9.1 Optical density

An Optical density test was applied every 24 hours to measure bacterial growth in the systems. The process has been mentioned in the section 2.2.7.

2.2.9.2 Dinitrosalicylic acid (DNS) test

A DNS test was applied every 24 hours to control the carbon source in the appropriate range for growing bacteria. The process has been mentioned in the section 2.2.8.

2.2.9.3 Chemical oxygen demand (COD)

Carbon source content in the systems was checked occasionally by measuring soluble COD in the systems. To analyze the COD using the HACH DR 3900 spectrophotometer and COD Digestion Vials, High Range, the following procedure was followed. The DRB200 Reactor was powered on and preheated to 150 °C. The samples were filtered using a Syringe Filter with a pore size of 0.45

µm. A vial for the selected range was uncapped and 2mL of the sample was added using a clean pipette. Another vial was uncapped, and 2 mL of deionized water was added as a blank. The vials were tightly closed, rinsed with water, and gently inverted to mix the contents. The vials were then placed in the preheated DRB200 reactor and heated for 2 hours. After heating, the reactor power was turned off, and the vials were allowed to cool for approximately 20 minutes to 120 °C or less. The cooled vials were gently inverted and placed in a tube rack to reach room temperature. The spectrophotometer program was started in the COD HR setting. The blank sample cell was cleaned and inserted into the cell holder, and the ZERO button was pressed to set the baseline at 0.0 mg/L COD. The prepared sample cell was cleaned and inserted into the cell holder, and the READ button was pressed to obtain the COD results in mg/L.

2.2.9.4 Nitrogen- ammonia

To determine the concentration of Ammonia Nitrogen using the HACH DR 3900 spectrophotometer and AmVer™ Salicylate Test 'N Tube™, the procedure outlined in the Hach Method 10031 was followed. The program "N, Ammonia HR TNT" was initiated on the DR 3900 spectrophotometer.

To prepare the samples, a syringe filter with a pore size of 0.45 µm was used to filter each sample. A blank was also prepared by adding 0.1 mL of ammonia-free water to a test tube. Next, 1 mL of the filtered sample was added to another test tube followed by the addition of 10 mL of Reagent 1 (AmVer™ Salicylate Reagent Powder Pillows). The test tube was then placed on a shaker for 5 minutes. After 5 minutes, 1 mL of Reagent 2 (AmVer™ Nitroprusside Reagent Solution) was added to the test tube and the tube was placed on a shaker for an additional 5 minutes. The completed test tube was then placed in the DR 3900 spectrophotometer and the absorbance of the solution was measured at 640 nm. The concentration of Ammonia Nitrogen was then determined

based on the calibration curve and reported in mg/L. This procedure was followed for all samples and the results were recorded for further analysis.

2.2.9.5 Total phosphorous

To determine the concentration of Phosphorus using the HACH DR 3900 spectrophotometer and Phosphorus (Reactive) TNT plus Vial Test, the following procedure was followed based on the Hach Method 10214. First, 5.0 mL of the sample was added to the test vial using a pipette. The vial was then tightly capped and inverted 2-3 times to mix the contents. The reaction was allowed to proceed for 10 minutes. After the timer expired, the vial was again inverted 2-3 times. The vial was then cleaned and subsequently inserted into the cell holder of the spectrophotometer. The results, expressed in mg/L of PO_4^{3-}, were obtained and displayed on the instrument.

Analyzing of biofilm formation: A variety of direct and indirect methods have been used to quantify cells in biofilms. Direct counting methods permit the enumeration of cells that can be cultured, including plate counts, microscopic cell counts, Coulter cell counting, flow cytometry, and fluorescence microscopy. Indirect measurement methods include the determination of dry mass, total organic carbon, microtiter plate assays, ATP bioluminescence, total protein, and quartz crystal microbalance (Wilson et al., 2017). In this experiment, to characterize biofilm, we used optical density, protein content, light microscopy, and scanning electron microscope (SEM).

2.2.9.6 Protein content

One widely accepted surrogate for total biofilm growth is total protein content. Assuming that protein content is approximately similar between cells, protein content has been found to correlate with the number of cells in biofilms in biofilms of wetland microcosms (Wilson et al., 2017). Synthetic water was employed as the ambient water in the systems, and there was no protein source present in the water before biofilm formation. Protein content in the water was measured every 48

hours to assess biofilm formation in the systems. The Modified Lowry Protein Assay Kit was utilized for quantifying protein contents in the systems.

For many years, Lowry's method was the most widely used and cited procedure for protein quantitation. The procedure involves the reaction of protein with cupric sulfate and tartrate in an alkaline solution, resulting in the formation of tetradentate copper-protein complexes. When the Folin-Ciocalteu Reagent is added, it is effectively reduced in proportion to these chelated copper complexes, producing a water-soluble product whose blue color can be measured at 750nm. There are two protocols to use the Lowry protein assay kit including "Test Tube Procedure" and "Microplate Procedure".

In the experiment, the test tube procedure specified in the instructions of the Modified Lowry Protein Assay Kit by ThermoScientific was employed. First, 40 μL of each standard and unknown sample replicate was pipetted into designated wells of a microplate. Subsequently, 200 μL of Modified Lowry Reagent was added to each well simultaneously, followed by immediate mixing on a plate mixer for 30 seconds. The microplate was then covered and incubated at room temperature for precisely 10 minutes. Afterwards, 20 μL of the prepared 1X Folin-Ciocalteu Reagent was added to each well, and the microplate was mixed again for 30 seconds. The plate was covered and incubated at room temperature for 30 minutes. The absorbance of the samples was measured at or near 750 nm using a plate reader. The average 750 nm absorbance value of the Blank standard replicates was subtracted from the 750 nm values of all other individual standard and unknown sample replicates. To determine the protein concentration of each unknown sample, a standard curve was prepared by plotting the average Blank-corrected 750nm values for each BSA standard against its concentration in μg/mL. It is recommended to utilize curve-fitting algorithms,

such as a four-parameter (quadratic) or best-fit curve, for more accurate results. Alternatively, a point-to-point curve can be used if plotting the results manually.

2.2.9.7 Light microscopy

Cell counting and biofilm morphology studies including two-dimensional surface structures can be accomplished using light microscopy. “Leica DM1000 Microscope” with a maximum magnification of 100X was used in this experiment.

Leica Image Organizer software, with an integrated database, is an easy-to-use tool for image archiving, particularly for clinical applications, and is also ideal for any field in which simple image storage and search options are required (product brochure).

2.2.10 Scanning electron microscopy (SEM)

In this experimental study, ThermoFisher Quanta 3D FEG was used for the SEM study. This is a DualBeam instrument that combines a traditional Field Emission column with a focused Ion beam (FIB) column to complement characterization laboratory tools and extend the application range to 3D characterization and nanoanalysis, transmission electron microscopy (TEM) sample preparation, or structural modification of sample surfaces at the nanometer scale.

This instrument features live SEM imaging while FIB milling, making it a superior solution for the fast preparation of large samples over a wide range of materials. It is now possible to increase sample throughput by using Quanta’s high-current FIB for fast material removal. An Omniprobe micromanipulator is available for in-situ TEM cross-section sample lift-out. Chemically enhanced etching can be completed of polymers, diamonds, oxides and metals, as well as biological samples.

Chapter Three: Results and discussion

An evaluation of the effects of the type of bacteria, nutrient, shaking velocity, and the size of the MPs was carried out in the batch system. The growth kinetics of bacteria in the ambient water as well as the rate of glucose consumption have been measured and discussed in this section 3.1 to determine the types of bacteria that can grow in our ambient water. In section 3.2, results and analysis are performed to determine the effect of nitrogen source as another main nutrient that can be utilized to promote the growth of bacteria.

There were six different types of bacteria isolated from wastewater (WW1, WW2, WW3, WW4, WW5, and WW6) and the results of these two sections reveal that three of the types (WW1, WW2, WW5) could grow in our particular ambient water, so DNA test was used to identify these three types and the results presented in section 3.3.

The effects of combinations of bacteria and the effect of change in shaking speed during the growth process, has been discussed in Sections 3.4 and 3.5 of this report.

Based on the results of the above experiments in the batch systems, continuous systems were set up in an optimized situation to study the effect of flow rate, and to do so, a lab-scale pilot was proposed to accelerate MPs' biofouling in the future. The results have been presented in sections 3.6 and 3.7.

Light microscopy and scanning electron microscopy methods were used to characterize the formation of biofilms on microplastic surfaces, and an indirect evaluation was performed by determining the protein content in the water, which has been explained in sections 3.7,3.8, and 3.9 of this chapter.

3.1 Kinetics results and glucose consumption by bacteria in ambient water

An optical density test was used to evaluate the bacterial growth and the DNS method was applied to determine the glucose consumption by six types of isolated bacteria. The results are shown in Figure 3-1.

Based on these results, the bacteria labeled WW1 shows the most rapid and consistent growth over the given time. It starts from a low optical density and increases steadily, indicating that this bacteria type has a strong ability to proliferate in the given conditions. In contrast, the bacteria labeled WW2, though starting at a higher optical density than WW1, also showed a notable increase over time, but its growth rate seems to level off after about 24 hours.

The WW5 bacteria had a more moderate growth rate compared to WW1 and WW2. It started growing slowly but showed a steeper increase after 15 hours, indicating a possible lag phase before entering a more rapid growth phase.

On the other hand, the bacteria labeled WW3, WW4, and WW6 appear to have either stagnant growth or a decline in optical density over time. This suggests that these bacteria might not be suited for the given conditions, or they have reached a stationary phase earlier than the others.

These observations align with the established understanding of bacterial growth dynamics. The varied responses of these bacteria to the same conditions highlight the diversity in bacterial adaptation and growth strategies

1. The WW5 bacteria have a more moderate growth rate compared to WW1 and WW2. It started growing slowly but showed a steeper increase after 15 hours, indicating a possible lag phase before entering a more rapid growth phase.
2. The bacteria labeled WW3, WW4, and WW6 appear to have either stagnant growth or a decline in optical density over time. This suggests that these bacteria might not be

suited for the given conditions, or they have reached a stationary phase earlier than the others.

Thus, WW1 has the most rapid growth, suggesting it is highly adaptable or beneficial under the tested conditions. WW2 also demonstrates adaptability, though its growth rate seems to level off after a certain period. WW5 has a lag phase, which is a period of slow growth, followed by a more rapid growth phase. WW3, WW4, and WW6 do not fare as well in these conditions based on their stagnant or declining optical density values. Thus, in ambient water, only three types of bacteria (WW1, WW2, and WW5) were grown. Thus, these three types of bacteria were selected to be tested for DNA. Table 3-1 presents the results.

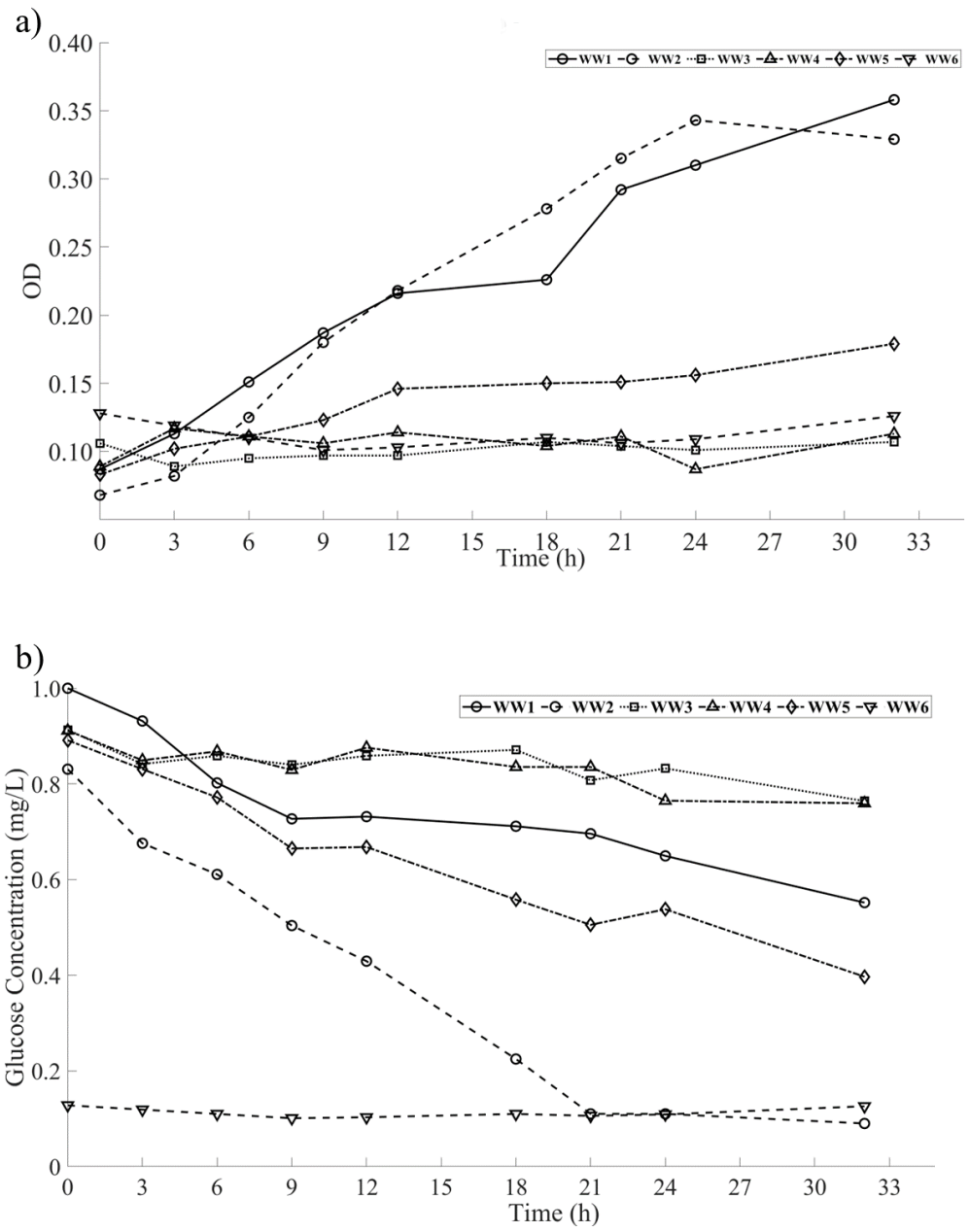
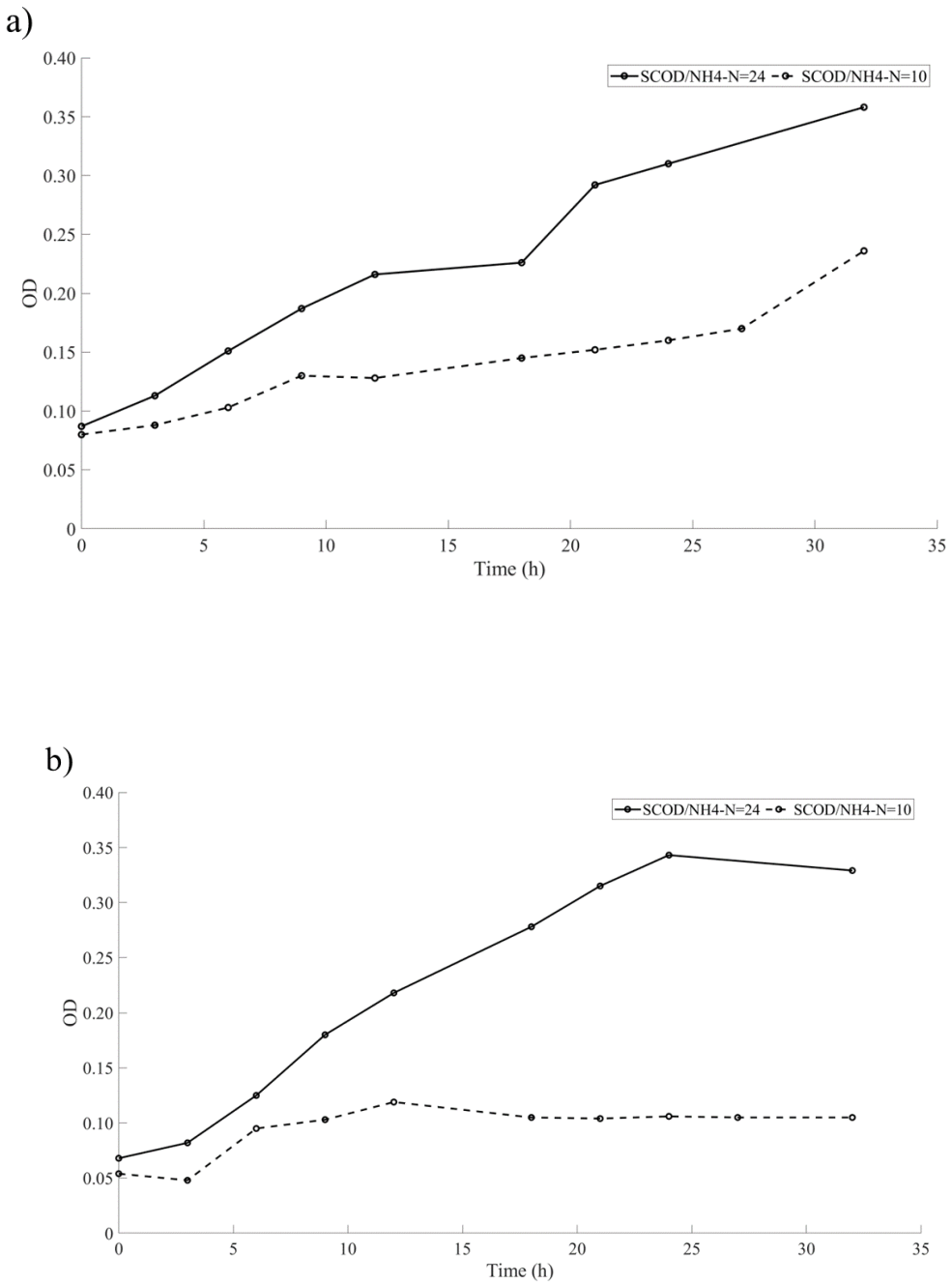


Figure 3-1 (a) Optical density and (b) Dinitrosalicylic acid results in ambient water including bacteria

3.2 Effects of extra nitrogen source

The experiments conducted by Lin et al. (2004) revealed that altering the ratio of soluble chemical oxygen demand (SCOD) to ammonia nitrogen from 10:1 to 6:1 led to nitrification. Nitrification,

in turn, was identified as an inhibitory factor for bacterial growth (Lin et al., 2004). In the ambient water, the ratio of SCOD/NH_4-N was changed from 24:1 to 10:1 to determine the effect of the carbon source to nitrogen source ratio as the main nutrients for the growth of three types of bacteria. The results are shown in Figure 3-2.



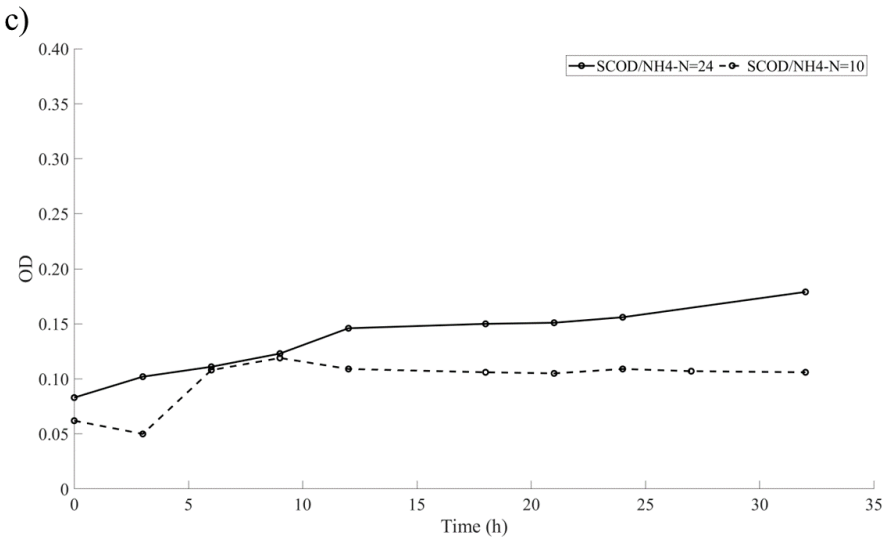


Figure 3-2 Effects of extra nitrogen source on bacterial growth -a) wastewater 1, b) wastewater 2, c) wastewater 5

As can be seen from the above figures, OD results do not improve with increased nitrogen levels in our systems. Other studies confirm that the optimal Carbon: Nitrogen (C: N) ratio varies by bacteria type. Panigrahi et al. (2018) found that C: N ratio level variations affect bacterial diversity and frequency distribution in water (Panigrahi et al., 2018).

Thus, despite the importance of nitrogen content to some types of bacteria, the tests indicated that additional nitrogen sources did not improve the OD results for the three types of bacteria that were studied so, the first ratio (SCOD/NH_4-N=24) was applied in the batch and continuous test in this study.

3.3 Bacterial identification

DNA tests were conducted to identify the types of bacteria growing in ambient water and their results were presented in Table 3-1.

Table 3-1 Result of DNA tests

Number	Bacteria Name	Match Percentage
WW1	*Pseudomonas fluorescens*	87%
WW2	*Comamonas thiooxydans*	95%
WW5	*Comamonas testosteroni*	96%

3.4 Bacterial growth in ambient water with different combinations of bacteria

To evaluate the effects of MPs size and mixing velocity in the batch systems, bacterial growth and biofilm formation were studied in the systems including small (355-425μm) and large MPs (1-2 mm).

Three types of bacteria that can grow in ambient water as well as their various combinations were tested. The combinations were T1 (WW1), T2 (WW2), T3 (WW5), T4 (WW1+WW2+WW5), T5 (WW1+WW2), T6 (WW1+WW5), and T7 (WW2+WW5). The mixing velocity increased from 30 to 180 RPM (revolution per minute) after 210 hours. The results are shown in Figure 3-3.

These figures demonstrate a high increase in OD results with increasing RPMs. This is because oxygen levels increased with rotation speed.

Using light microscopy, the surface of small and large MPs was studied to determine how their size affected biofilm formation. The section 3.5 presents these results.

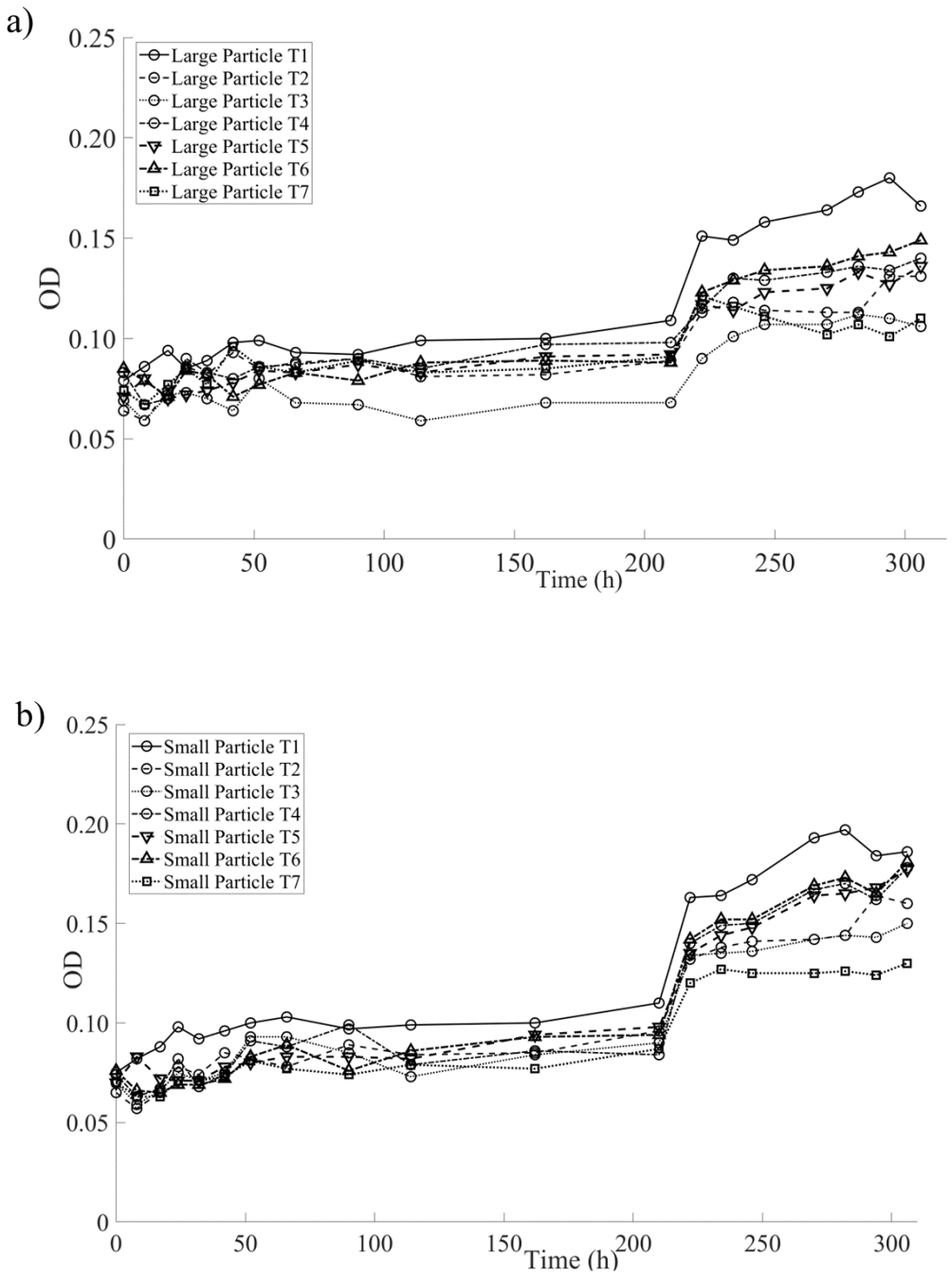


Figure 3-3 Optical density results for different combinations of bacteria in the ambient water including a) large MPs and b) small MPs

3.5 Microscope study on the effects of MPs' size on biofilm formation

The use of visual microscopy, especially when combined with staining techniques, offers a direct and effective method for biofilm quantification, as supported by numerous studies in the literature (Nag, 2021).

Changes on the surface of large and small MPs were studied by using light microscopy with 40 X magnification after two weeks. Both were chosen from T1 and compared with virgin MPs. The results are presented in Figure 3-4 .

As is shown in these figures due to their higher specific surface area, small MPs have more biofilm on their surfaces.

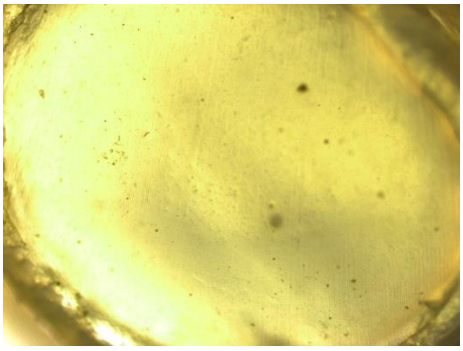
The ambient water containing these MPs has also been subjected to microscopy studies after two weeks. The results with a magnification of 100X are presented in Figure 3-5 .

In these pictures, there is a much higher colonization of bacteria which could be a sign of biofilm formation in the batch system that includes small MPs while there is a much lower colonization in the system which includes large MPs.

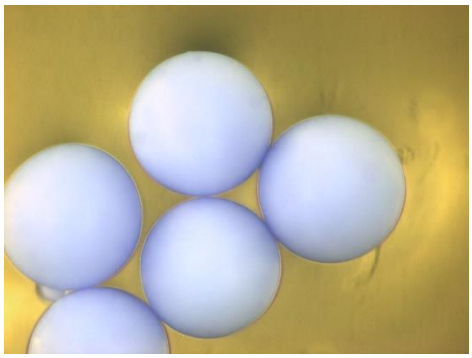
Considering these results, small orange polyethylene MPs with a size of 106-125 µm were selected, because it was easy to visually monitor their surface changes on continuous systems.

Virgin MPs MPs in the ambient water

a)



b)



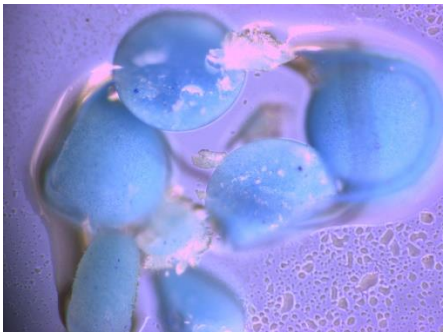
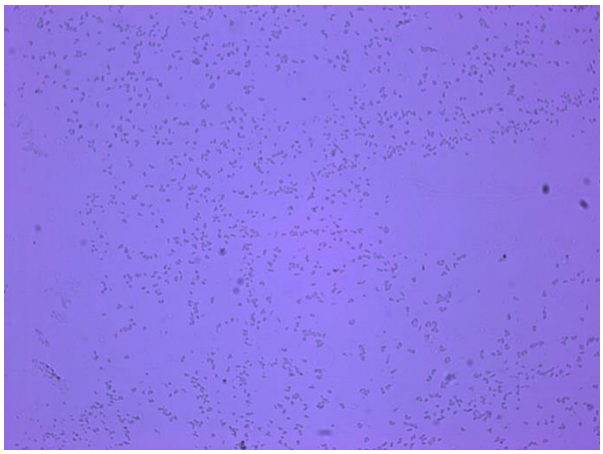
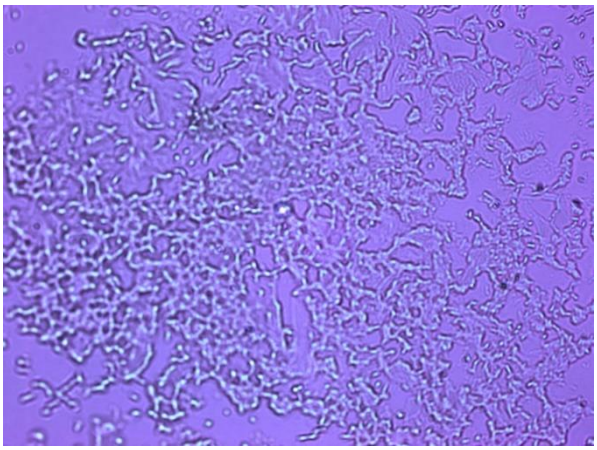


Figure 3-4 Comparing surfaces of virgin MPs (right) and biofouled MPs surface in the batch systems after two weeks: a) large MPs: no specific changes can be seen on the surface and b) small MPs: surface changes could be the result of biofilm formation.





(a) (b)

Figure 3-5 Comparing ambient water at t=0 and t=two weeks in the batch systems) large MPs: without any bacterial colonization and b) small MPs: colonization of bacteria in the ambient water could be the result of biofilm formation in the system

3.6 Effects of flow rate on bacterial growth in the continuous systems

In a continuous set-up, three continuous systems with MPs were started to work at the same time and in the same environment. Each system had a different flow rate: 65 mL/min for the first system, 50 mL/min for the second system, and 35 mL/min for the third system. The glucose concentration in all the systems was 2g/L, and the SCOD to NH_4-N ratio was 24 in each system. Each system had a 10% v/v of *Pseudomonas* bacteria with an OD=1.

Optical Density was measured every day to evaluate bacterial growth in these three systems. The results are shown in Figure 3-6.

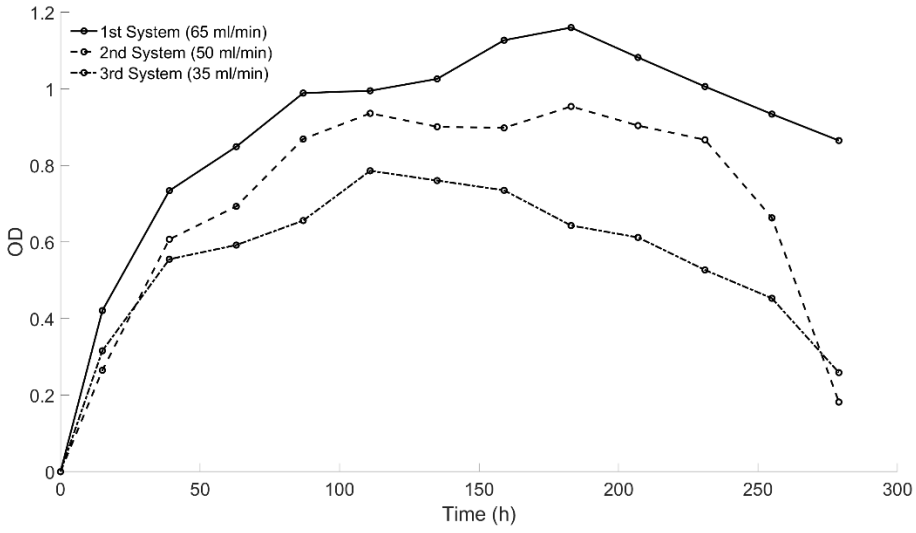


Figure 3-6 Comparing OD results in three continuous systems

It can be seen from Fig 3-6 that bacteria grew faster when the flow rate of recirculation was increased in continuous systems. There could be a higher oxygen concentration in systems with a higher recirculation flow rate that could be responsible for this. The effect of flow and peristaltic mixing in channels has already been studied and the results demonstrated that the physical forces of flow and mixing could have a significant effect on bacterial growth and ecology (Cremer et al., 2016).

Following ten days of continuous operation, light microscopy and scanning electron microscopy were employed to compare the formation of biofilm on the surface of MPs in three systems. Results are presented in sections 3.7 and 3.8.

3.7 Microscope study on the effects of flow rate on biofilm formation

After ten days of operation of the continuous systems, microscopic studies were conducted on the surface of MPs by magnification of 40X. By using a light microscope at a magnification of 100X, the ambient water in three systems was also examined to compare bacteria colonization in the water. The results are presented in.

Compared to the two other systems, fewer changes can be seen on the surface of MPs in the first system. There is a possible conclusion to be drawn from the study that, although bacteria grew so quickly in the system with the highest flow rate (based on the results of the section 3.6), they did not have enough time to attach themselves to the surfaces of the MPs, as shown in the study.

This conclusion was further confirmed when comparing the bacteria colonization in the ambient water. As can be seen Figure 3-7, the bacteria colonized less in the first system that was shown to have less amount of biofilm than in the second system.

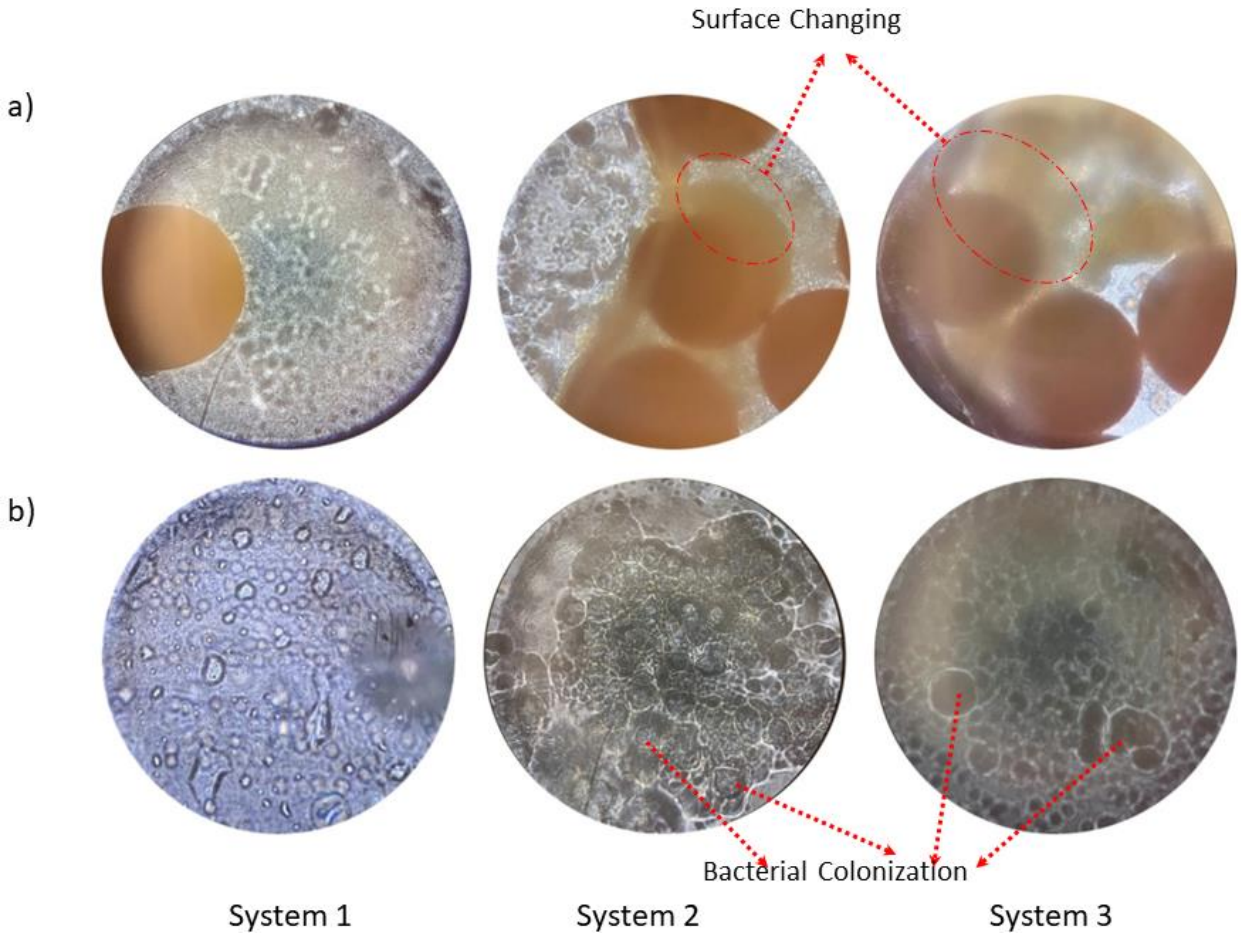


Figure 3-7 (a) MP's surface changes: no specific changes in the first system, but MP's surface in two other systems changed, possibly because of biofouling. and (b) Ambient water of three continuous systems after 10 days shows more bacterial colonization in 2nd and 3rd system which could be the result of biofouling

Zhang, et al. showed the same result in their study on the effects of pore-scale heterogeneity and transverse mixing on bacterial growth in porous media. They demonstrated that biomass grows preferentially in pore bodies where the flow rate is slower and there is less shear force (C. Zhang et al., 2010).

A detailed analysis of the changes on the MP's surface caused by biofouling was carried out using SEM, and the results are presented in the section 3.8.

3.8 SEM results on MPs surfaces before and after biofouling

To examine the surface changes of MPs after 10 days in the three continuous systems, a scanning electron microscope with magnifications of 1000 X and 5000 X was used. The results are presented in Figure 3-8 and Figure 3-9 . As illustrated in these figures, the textures of biofilms on the surfaces of the MPs were different, As a result, the composition of the biofilm changed considerably over time. As can also be seen from these figures, the biofouling process in the first system was slower and some parts of the MP's surface in this system are still uncovered and have the same consistency as the virgin MP's surface.

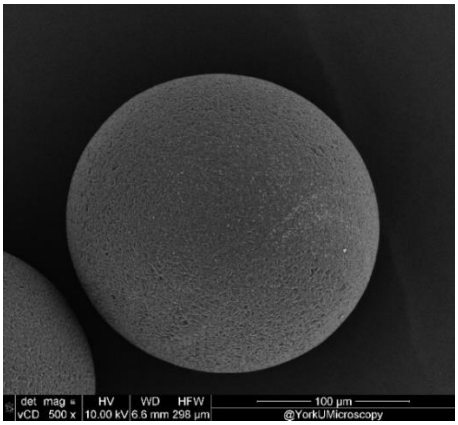
The comparison to the virgin MP surface provides a baseline to understand how much the MP’s surface has been altered by the biofilm. The uncovered regions on the MP’s surface in the first system, which resemble the virgin MP surface, suggest that the environment in this system might be less conducive to rapid biofilm development.

In the first system, a slower biofouling process with some parts of the MP's surface still resembling the virgin MP was observed. This happens due to several reasons:

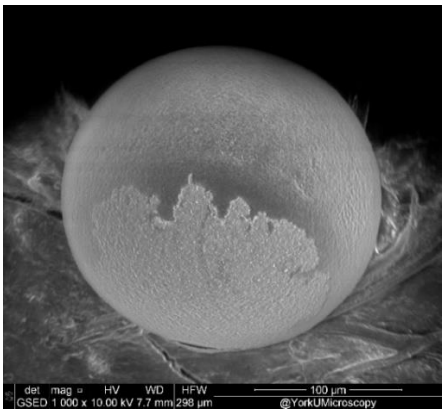
- Higher shear forces in the system prevent bacteria from effectively adhering and proliferating on the MP's surface.
- Nutrient availability was different, affecting the rate at which bacteria can colonize and form biofilms.
- Other microbial interactions, such as competition or predation, also play a role in influencing the biofouling rate.

For our set-up, protein content tests were conducted to determine what caused the slower biofouling. Whether defouling occurred in the first system or if biofouling exhibited reduced rates

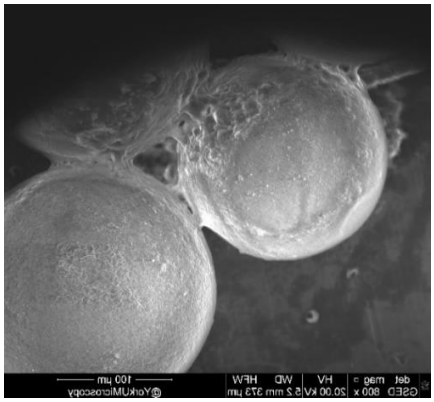
due to factors such as diminished nutrient availability or other microbial interactions can be determined by measuring protein content in the ambient water across all systems.

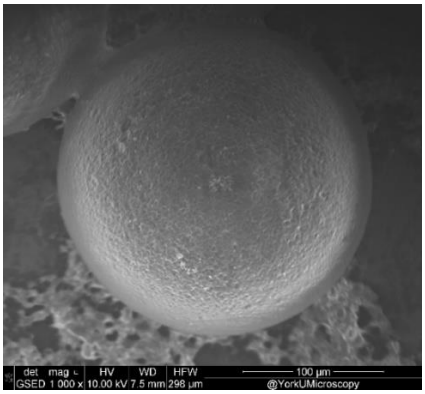


Virgin MP

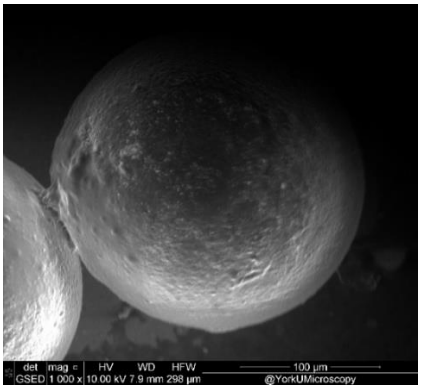


1st System



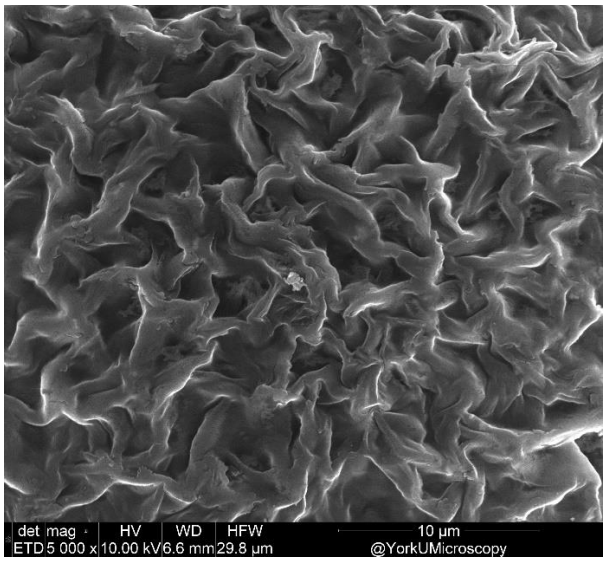


2nd System

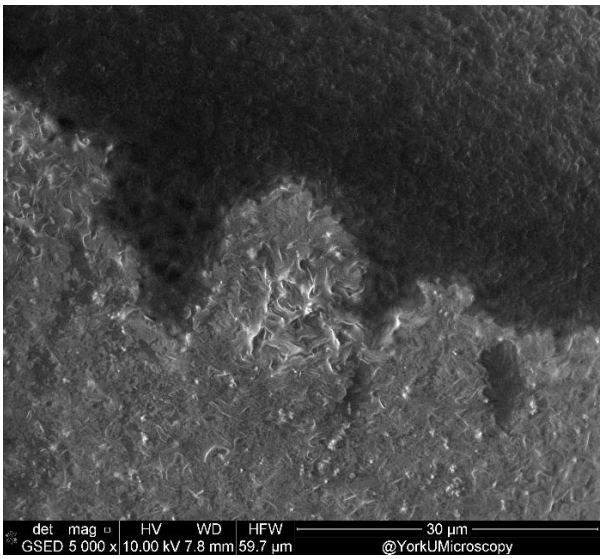


3rd System

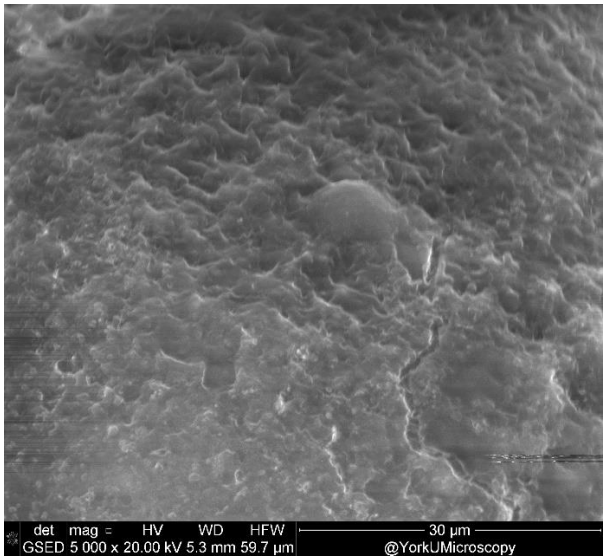
Figure 3-8 SEM results on MPs surfaces before and after biofouling (1000X). a) shows MP's surface without any biofilm. There are some changes in MP's surface compared to virgin MP, while others remain the same. c) and d) MPs' surface is completely covered by biofilm



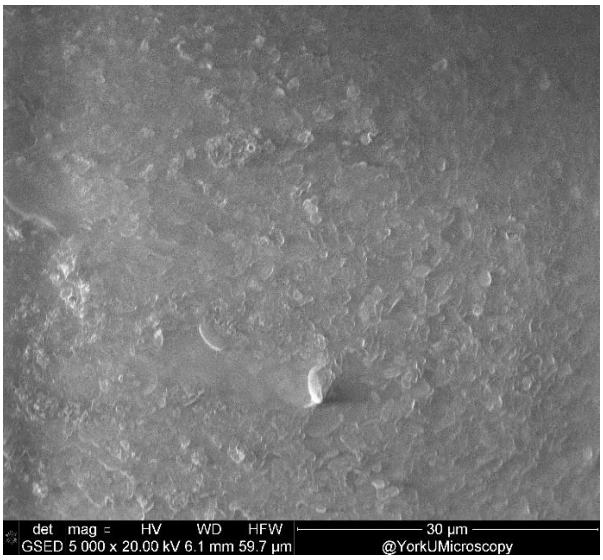
Virgin MP



1st System



2nd System



3rd System

Figure 3-9 SEM results on MPs surfaces before and after biofouling (5000X magnification). a) shows MP's surface without any biofilm. There are some changes in MP's surface compared to virgin MP, while others remain the same. c) and d) MPs' surface is completely covered by biofilm

3.9 Protein content in the ambient water

To characterize biofilm formation in the systems "total protein content" was measured. One widely accepted surrogate for total biofilm growth is total protein content. Protein quantification is a quick, commonly available assay which allows for a relative assessment of biofilm growth. This method was not applicable directly in our experiments, because of the size of MPs and low amount of biofilm from start-up of the system until 10 days. As the protein content of the synthetic water in this study was initially at zero, it was possible to measure it in the supernatant every 48 hours instead of on the surfaces of MPs to quantify the biofilm formation in the systems. Figure 3-10 shows the results of the protein content test in three systems.

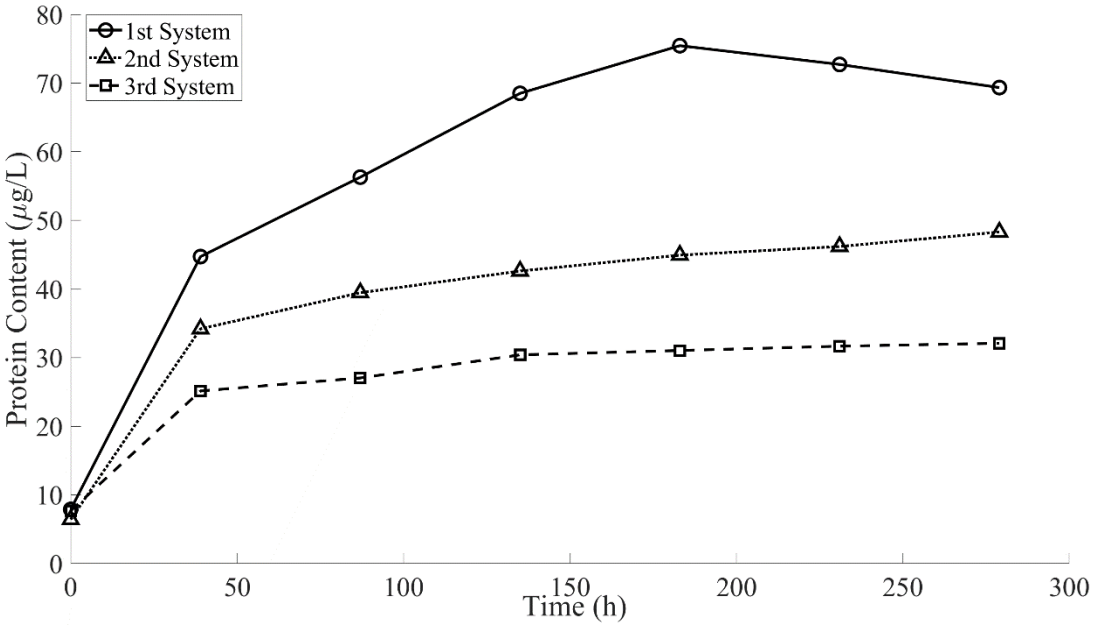


Figure 3-10 Protein content in three systems with three different flowrates (1st: 65 mL/min; 2nd: 45 mL/min; 3rd: 60 mL/min) versus time

A complex 3-D architecture of biofilm is started by developing a hydrogel-like matrix containing EPS on the surface of MPs. EPS are biopolymers that consist of polysaccharides, proteins, nucleic acids, lipids, and humic substances (Flemming, 2002). Thus, protein content increased

significantly along the biofilm growth (Chao and Zhang 2012). Based on the protein test results, in all three systems, protein content increases over time which demonstrates biofilm formation in the systems. As shown, the protein content in the first system is higher than two other systems. When these results were combined with microscopic studies (Figure 3-7) that showed fewer changes in the surface of MPs in the 1st system, it was easily found that microorganisms do not have enough time to attach to the MPs. These results showed that although protein content in the 1st system ambient was higher, colonization of the microorganisms was less than in other systems. It could also be a sign of biofilm detachment from the MP surface. As discussed in other studies, the initial step of biofouling is reversible attachment (Flemming, 2002), hence it could be concluded that biofilm formation in the 1st system was in the initial stage and a higher flow rate caused higher shear stress and eventually caused detachment of biofilm from the MPs' surface.

Biofilm formation progresses through multiple developmental stages, beginning with reversible and then irreversible attachment of cells to a surface, followed by the formation of microcolonies, the maturation and differentiation of the biofilm with the expression of matrix polymers, and finally dispersal of cells from the biofilm (Stoodley et al. 2002; Sauer et al. 2004). Extracellular polymeric substances (EPS), including proteins, polysaccharides, and extracellular DNA (eDNA), can be involved in attachment processes during the initial stages of biofilm formation (Tielen et al., 2010) that is an irreversible attachment and could be detached from the MPs' surface with shear stress due to high flow rate in the first system. Thus, the protein content in the first system's ambient water was higher than in other systems, however, biofilm formation and biomass colonization in this system were lower than in others.

Chapter Four: Conclusion and future work

4.1 Conclusion

A comprehensive investigation of both batch and continuous experimental setups uncovered an understanding of the intricate interactions between bacterial communities, nutrient dynamics, selective MP attributes, and flow conditions.

Bacterial growth and nutrient utilization: Our investigation focused on the kinetics of bacterial growth and glucose consumption among a variety of bacterial strains, extracted from wastewater sources. In our unique environmental water conditions, only three bacterial strains were able to thrive, namely *Pseudomonas fluorescens*, *Comamonas thiooxydans*, and *Comamonas testosteroni*. This strongly echoes this revelation in studies conducted by (Lin et al. 2004), which highlighted the crucial role of nutrient ratios in determining bacterial proliferation.

Effects of extra nitrogen source: Further exploration of nutrient dynamics led us to investigate how altered carbon-to-nitrogen ratios affect bacterial growth. For our selected strains, manipulating this ratio did not produce the expected surge in bacterial proliferation. The phenomenon aligns with (C. Zhang et al. 2010) who highlighted the complex interactions between bacterial growth and nutrient availability, hinting at the intricate balance within biofilms.

Bacterial identification: It was crucial to accurately identify bacteria to weave an intricate narrative about biofouling dynamics. Our selected bacterial strains had their true identities revealed through DNA testing and sequence analysis, a crucial basis for further studies.

Bacterial growth on MPs in batch systems: Bacterial growth on the surface of MPs was studied in the batch system. A single bacterial strain, *Pseudomonas fluorescens* (T1), exhibited the highest growth rate, highlighting the importance of competitive dynamics. The lack of competition

facilitated this bacterial strain's dominance, which aligns with Sadowska et al. (2010) 's notion of bacterial competition.

Continuous systems and flow rate effects: By switching to continuous systems, the effects of flow rate on bacterial growth were studied. Bacterial growth was facilitated by faster flow rates because there was more oxygen, (Cremer et al. 2016). While this reduction in biofilm formation might seem counterintuitive, it highlighted the delicate balance between bacterial attachment and detachment, similar to what Zhang et al. (2010) found with pore-scale heterogeneity.

Biofilm characterization and protein content: The textures of the biofilms on the surfaces of MPs with different flow rates were different based on SEM analysis. As a result, the composition of the biofilm changed considerably over time. There was a slower biofouling process in the higher flow rate system and some of the MP's surface remained uncovered and had the same consistency as the virgin surface.

Protein content increased over time in all three systems based on the protein tests. The results proved that biofilms form in the systems. Protein content was higher in the system with a higher flow rate than in the two other systems. In combination with microscopic studies, these results indicated microorganisms did not have enough time to attach to MPs in this system.

The results highlighted the intricate relationships among bacterial communities, nutrient dynamics, microplastic properties, and flow conditions, emphasizing the multifaceted nature of biofilm formation. This study emphasizes the need for more research, cooperation, and policies to tackle the issue of microplastic pollution and protect the balance of aquatic ecosystems.

4.2 Limitation of these Experiments

In this experimental study, certain limitations prevented the full replication of real-world environmental and physical conditions. The first notable limitation was time constraints. The study

only focused on the initial stage of biofouling, known as the conditioning film stage, where bacteria irreversibly adhere to a surface. This limitation led to the use of a simplified synthetic ambient water containing only nutrients and a single type of bacteria. Had there been more time, a more complex environmental matrix could have been created to observe the attachment of various species, like microalgae or different bacteria, and to study the sequence of their attachment.

Another limitation was the singular focus on one shape and type of microplastics (MPs). Specifically, spherical polyethylene MPs of identical size and other physical characteristics were used. These MPs were buoyant in the ambient water used in the experiment and had limited exposure to the mixture. This limitation meant that the study could not explore the effects of MPs with varying shapes and densities, which would have provided insights into how these physical characteristics influence biofouling processes.

Additionally, the experimental study implemented a closed system to prevent the entry of contaminants. This approach, while effective in maintaining the purity of the system, introduced another limitation: the inability to perform sampling during the process. As a result, the researchers could only collect samples at two points in the study - the initial stage before biofouling began and the final stage, which was after 10 days. This limitation meant that the study could not provide continuous data on the progression of biofouling over time, offering insights only at the beginning and end of the 10 days. This gap in data collection restricted the ability to observe and analyze the intermediate stages of biofouling, potentially overlooking significant changes or trends that may have occurred during the process.

4.3 Future recommendations

To evaluate the biofilm formation on MPs, we had to narrow down our objectives and develop research questions. In the process, simplifications are made in experimental design, which has to

be considered in interpreting the outcomes. For instance, only one type of bacteria, Pseudomonas, was used in the continuous setup and the study didn't look at how different kinds of bacteria might interact under various flow conditions. A single type of microplastic was also studied in this study. It didn't explore how microplastics with different surface properties and shapes might affect the results.

Furthermore, this study focused only on the effects of main nutrients like glucose and nitrogen. Additionally, the effect of limited environmental factors was considered in the study, while the effect of others, including UV radiation and water depth, was not examined. Based on these limitations and our findings, the following recommendations are made for future research on microplastic biofouling:

UV light: The influence of UV light on microplastic biofouling remains an intriguing puzzle awaiting exploration. UV radiation possesses the potential to disrupt microbial communities, alter biofilm development, and even trigger chemical transformations on microplastic surfaces. Future investigations could unravel the intricate mechanisms underlying these phenomena. By subjecting microplastic-biofilm assemblages to controlled UV exposures, researchers can probe how UV wavelengths impact bacterial composition, biofilm structure, and the degradation of both plastic and associated biofilm matrices.

Water depth: Future studies should give greater consideration to the role of water depth in aquatic systems concerning the biofouling of microplastics. Within various depths of water bodies, environmental parameters shift, affecting nutrient availability, light penetration, and hydrodynamic conditions. In the future, the dynamics of biofilm formation across depth gradients could be investigated, shedding light on how bacterial communities adapt to varying conditions.

Such research may uncover vertical variations in biofilm composition, growth rates, and interactions, emphasizing the importance of depth as a determinant of the biofouling process.

Hybrid impacts of UV light and depth synergy: The intersection of UV light exposure and depth gradients presents an intriguing arena for research synergy. How do these factors synergistically shape biofouling phenomena? Could UV-induced alterations in microbial communities differ across different depths? Exploring these questions could lead to insights into how varying UV intensities, coupled with depth-related environmental shifts, collectively orchestrate biofilm development. Such revelations could have implications for the design of UV-based biofouling management strategies across diverse aquatic ecosystems.

Microbial diversity and dynamics: This study revealed how bacteria behave on microplastics, but there's still much to learn about how different types of bacteria interact, especially in biofilms. Studying these various bacteria further could help us understand more about biofouling, which is when surfaces get covered by microorganisms.

Nutrient interplay and adaptation: The symphony of nutrient dynamics and its interplay with bacterial growth warrants a more detailed exploration. Future studies could delve into the adaptations exhibited by bacterial communities when exposed to varying nutrient ratios and conditions. Understanding how microbial communities respond and adapt to changing nutrient landscapes could unravel the intricate tapestry of nutrient-driven biofouling scenarios, echoing the adaptive strategies observed in various ecological systems.

Complex microplastic surfaces: This study focused on the interaction between bacterial communities and microplastics of specific sizes and attributes. Exploring the influence of diverse microplastic surface properties—such as texture, composition, and chemical modifications—could unlock a wealth of insights. Future research could investigate how these attributes shape bacterial

attachment, biofilm formation, and overall biofouling dynamics. A nuanced understanding of these interactions could pave the way for tailored strategies to mitigate microplastic-associated biofouling.

Dynamic flow conditions: The interplay between flow dynamics and biofilm development showcased an intriguing paradox. The increased bacterial proliferation with higher flow rates, coupled with reduced biofilm formation, presents an enigma worthy of further scrutiny. Future investigations could delve deeper into the underlying mechanisms of this phenomenon, considering factors such as shear stress, attachment kinetics, and biofilm stability. This exploration could provide valuable insights into harnessing flow dynamics for effective biofouling management.

Integration of advanced imaging techniques: While this study harnessed microscopy to visualize biofilm development, the integration of advanced imaging techniques could elevate the depth of our understanding. Techniques such as confocal laser scanning microscopy (CLSM) and time-lapse imaging could offer real-time glimpses into the intricate dynamics of biofilm formation. These tools could provide invaluable visual narratives of the biofouling journey, capturing transitions, growth kinetics, and detachment events in unprecedented detail.

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