IDENTIFICATION OF MICROPLASTIC AND MICROBIAL COMPOSITIONS IN A WASTEWATER TREATMENT PLANT EMPLOYING RBC UNIT

by

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B.Sc. in Environmental Engineering, Istanbul University, 2018

Submitted to the Institute of Environmental Science

in partial fulfillment of the requirements for the degree of

Master of Science

in

Environmental Technology

Boğaziçi University

2025

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DATE OF APPROVAL: 17/12/2025

ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere gratitude to my supervisor, Prof. Dr. Burak Demirel, for his guidance, support, and valuable feedback throughout this study. I am also deeply thankful for his guidance on matters beyond the scope of this thesis. I would also like to thank Research Assistant Suat Vardar, whom I consulted frequently during the course of this research, for his continuous support, insightful discussions, and practical guidance.

I am grateful to Assoc. Prof. Dr. Berat Haznedaroğlu for kindly allowing me to use his laboratory facilities, particularly the stereomicroscope and Raman spectroscopy equipment. I would also like to thank Assoc. Prof. Dr. Ulaş Tezel and Prof. Dr. Bahar İnce for granting access to their laboratories and providing laboratory equipment that supported different stages of this work. I would also like to express my sincere thanks to Prof. Dr. Arzu Çelik from the Department of Molecular Biology and Genetics for her support and for kindly allowing the use of their microscopy facilities.

For the microbial analyses, I would like to thank the Research Assistants Ömer Uzun, Recep Can Altınbağ, and Koray Sakarya for their technical assistance and support. I am grateful to my dear friend Berivan Boz for her constant support during my time at the institute and for her wonderful friendship. I also thank Dr. Havva Ateş for her support and encouragement during moments when I doubted myself, and for the inspiration and clarity she helped me regain.

This study was financially supported by Boğaziçi University Scientific Research Projects (BAP) under Project No. 19870, and this support is gratefully acknowledged.

Finally, I would like to express my deepest gratitude to my parents, Zeynep and Mehmet, for their endless love, support, and encouragement throughout my academic journey and beyond. Their constant presence, trust, and emotional support have been a steady source of my motivation and resilience, accompanying me at every stage of this challenging process.

ABSTRACT

IDENTIFICATION OF MICROPLASTIC AND MICROBIAL COMPOSITIONS IN A WASTEWATER TREATMENT PLANT EMPLOYING RBC UNIT

This study investigates the abundance, characteristics, removal efficiency, and microbial colonization of microplastics (MPs) in a full-scale wastewater treatment plant (WWTP) employing Rotating Biological Contactor (RBC), an attached-growth technology for biological treatment. Wastewater and sludge samples were collected in three seasons (winter, spring, and summer), and a total of 6,160 suspected particles were isolated. Fibers were consistently the dominant morphology across all sampling points and seasons, accounting for 92% of all extracted particles. Seasonal variations were observed in influent concentrations, with the highest MP loads measured during winter and summer, likely corresponding to fluctuations in campus-related human activity patterns. Despite these variations, effluent concentrations remained low (12–14 particles/L), and overall removal efficiencies ranged between 92% and 96%. Sludge was identified as the major sink for MPs, containing 102–132 particles/g. Size-based evaluations revealed that medium-sized particles (250–1000 μm) were the predominant class and were removed more effectively than other fractions. Raman spectroscopy identified polyethylene terephthalate, polyvinyl chloride, nylon, and polypropylene as the most common polymer types. To assess microbial community structure, 16S rRNA gene sequencing was applied to both bulk samples and microplastic-associated fractions collected in November 2024 and February 2025. The dominant bacterial phyla across all samples were *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Chloroflexi*, although their relative abundances varied by sampling point and date. MP-associated microbial assemblages differed from the surrounding bulk communities, reflecting selective colonization processes on microplastic surfaces.

ÖZET

DÖNER BİYOLOJİK KONTAKTÖR TİPİ ARITMA TESİSLERİNDE MİKROPLASTİKLERİN AKIBETİ VE MİKROPLASTİK VARLIĞINDA BİYOLOJİK KOMÜNİTELER

Bu çalışma, döner biyolojik kontaktör (RBC) kullanan tam ölçekli bir atıksu arıtma tesisinde (AAT) mikroplastiklerin (MPs) bolluğunu, özelliklerini, giderim verimini ve mikrobiyal kolonizasyonunu incelemektedir. Atıksu ve çamur örnekleri üç farklı mevsimde (kış, ilkbahar ve yaz) toplanmış ve toplam 6.160 şüpheli parçacık izole edilmiştir. Lifler, tüm örnekleme noktaları ve mevsimlerde tutarlı biçimde baskın morfoloji olarak görülmüş ve tüm parçacıkların %92'sini oluşturmuştur. Giriş suyu konsantrasyonlarında mevsimsel değişiklikler gözlenmiş, en yüksek MP yükleri kış ve yaz dönemlerinde kaydedilmiştir; bu durumun kampüs kaynaklı insan faaliyetlerindeki dalgalanmalarla ilişkili olabileceği düşünülmektedir. Bu değişimlere rağmen çıkış suyu konsantrasyonları düşük kalmış (12–14 parçacık/L) ve toplam giderim verimleri %92 ile %96 arasında gerçekleşmiştir. Çamur, 102–132 parçacık/g içeriğiyle MP'lerin başlıca birikim noktası olarak belirlenmiştir. Boyut temelli değerlendirmeler, orta büyüklükteki parçacıkların (250–1000 μm) baskın sınıf olduğunu ve diğer fraksiyonlara kıyasla daha etkin şekilde uzaklaştırıldığını göstermiştir. Raman spektroskopisi, polietilen tereftalat, polivinil klorür, naylon ve polipropülenin en yaygın polimer türleri olduğunu ortaya koymuştur. Mikrobiyal topluluk yapısını değerlendirmek amacıyla, Kasım 2024 ve Şubat 2025'te toplanan hem toplu örneklere hem de mikroplastiklere bağlı fraksiyonlara 16S rRNA gen dizilemesi uygulanmıştır. Tüm örneklerde baskın bakteriyel filumlar *Proteobacteria*, *Firmicutes*, *Bacteroidetes* ve *Chloroflexi* olmuş, ancak göreceli bollukları örnekleme noktasına ve tarihe göre değişmiştir. Mikroplastiklere bağlı mikrobiyal toplulukların çevresindeki genel topluluklardan farklılık göstermesi, mikroplastik yüzeylerinde seçici kolonizasyon süreçlerini yansıtmaktadır.

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LIST OF SYMBOLS/ABBREVIATIONS

Abbreviation	Explanation
ARB	Antibiotic resistant bacteria
ARGs	Antibiotic resistant genes
ATP	Adenosine triphosphate
BPA	Bisphenol A
EPS	Extracellular polymeric substances
HDPP	High-density polypropylene
HRT	Hydraulic retention time
MPs	Microplastics
PA	Polyamide
PAH	Polycyclic aromatic hydrocarbons
PE	Polyethylene
PES	Polyester
PET	Polyethylene terephthalate
POPs	Persistent organic pollutants
PP	Polypropylene
PPA	Polyphthalamide
PPSU	Polyphenylsulfone
PS	Polystyrene
PTFE	Polytetrafluoroethylene
PVBP	Poly(4-vinylbiphenyl)
PVC	Polyvinylchloride
RBC	Rotating Biological Contactor
WPO	Wet Peroxide Oxidation
WWTP	Wastewater Treatment Plant

1. INTRODUCTION

Industrialization, globalization, and population growth have increased the demand for commercial materials used in production. However, natural resources alone are insufficient to meet the growing need for industrial production. As a result, industries have turned to alternative materials that are cheaper, more flexible, and easier to manufacture. Plastics are one of those materials that are used heavily in industries. Due to their durability, strength, versatility, and cost-effectiveness, plastic materials have become deeply integrated into modern society (Chen et al., 2021).

According to Plastic Europe's latest report, 413.8 Mt of plastic was produced worldwide in 2023, and only 8.7% of them were produced from bio-based or recycled materials. Nearly three generations of usage, and without proper management of plastic waste, some scientists argue that the current period of our time will be called the "Plastic Age", due to the distinct plastic layer accumulating in the environment (Porta, 2021). The same properties that make plastics desirable, durability, chemical stability, and resistance to degradation, also make them environmentally persistent and hazardous. Once they emerge in a natural system, it becomes difficult to intervene, and the long degradation time of plastics leads to significant accumulation, causing a significant pollution in the receiving environment (Chen et al., 2021). Plastic pollution is a serious problem, especially in aquatic systems. Plastics can end up in aquatic systems via various routes (Cole et al., 2011). One of the routes is wastewater treatment plants (WWTPs), which are acknowledged as one of the major sources of plastic transportation in aquatic environments (Sol et al., 2020). Other routes are urban runoff, public littering, road surfaces, and more (Yaseen et al., 2022).

Plastic particles with sizes between 5 mm and 1 μm are called microplastics, and they are classified as primary and secondary microplastics based on their origin (Vardar et al., 2021). Primary microplastics refer to plastics that are purposely produced in micro sizes, while secondary microplastics form from the breaking down of bigger plastic materials (Ajibade et al., 2020). Personal care products, pellets, and pharmaceuticals are examples of primary microplastics (Ajibade et al., 2020). Fibers from washing synthetic clothes, automobile tires, and household materials can be examples of secondary microplastics (Hamidian et al., 2021).

In wastewater treatment plants, microplastics can be found in all stages of treatment steps. An important portion of microplastics is removed in the WWTP during treatment stages; however, WWTPs are not particularly designed for microplastic removal, and due to high daily loads of WWTPs, a huge part of microplastics continues to be discharged into the receiving environments through effluent waters (Franco et al., 2021; Le et al., 2023; Prajapati et al., n.d.). MP's removal efficiency differs according to the treatment technology as well as microplastics' shape and size (Lares et al., 2018). Moreover, some studies have shown that microplastics removed from wastewater tend to accumulate in the sludge, raising concerns about their impact on the environment due to their high amounts during management of WWTP sludge (S. Liu et al., 2023; Sun et al., 2019).

Another concern regarding microplastic pollution is their potential role as vectors for pathogens and carriers of antibiotic resistance genes (Martínez-Campos et al., 2021). Microplastics' hydrophobic surfaces enable microorganisms to easily attach to it and formation of biofilm. Moreover, the persistent nature of microplastics allows these colonized microplastics to spread across diverse environments. As a result, microplastics become a worrisome not only for environmental health but also for human health (Nguyen et al., 2023).

Most studies investigating microplastic fate in WWTPs focused on conventional WWTPs, which employ suspended growth processes; meanwhile, research on attached growth systems remains very limited as far as encountered in the literature. Although a few studies have examined microplastic behavior in attached-growth systems, such as trickling filters or RBCs, detailed assessments of microplastic fate within these systems are still scarce. A Rotating Biological Contactor (RBC) is an attached growth system used in biological wastewater treatment, consisting of several circular disks, shafts, drive systems, enclosures, and a settling tank (Irfan et al., 2022). RBC units are known and widely used for their low energy consumption, simple structure design, and low operation costs (Irfan et al., 2022; Waqas et al., 2021).

This study aims to demonstrate the stepwise removal efficiency of microplastics in a WWTP employing an RBC unit, thereby highlighting the role of the attached-growth process in microplastic retention. In addition, while many studies utilized laboratory simulations regarding microbial colonization of microplastics, this research characterizes microbial assemblages on the surface of microplastics directly obtained from a full-scale WWTP. By combining these two perspectives, the study

provides new insights into understanding the real-life interactions of microplastics and microorganisms in a WWTP, as well as the removal efficiency of the attached growth system on those particles.

2. LITERATURE REVIEW

2.1. Plastics

The first appearance of plastics in history dates back to 1600 BC with the production of rubber balls; however, the first fully synthetic plastic material was invented in 1907 (Stanley et al., 2025). Before the advent of fully synthetic plastics, several intermediate developments shaped plastic history. In 1862, Parkesine, known as the first thermoplastic, was presented at the Great International Exhibition by the inventor, Alexander Parkes, as a material that stands out with its flexible feature (Rasmussen, 2021). Following this, John Hyatt discovered a new material, Celluloid, an alternative for ivory to produce billiard balls using Parkesine in his experiments in 1869 (Streit-Bianchi et al., 2020). However, in 1907, the development of a new material called Bakelite, which is known first synthetic plastic, marked the beginning of modern plastics (Khan & Iqbal, 2023).

Plastics are synthetic materials composed of multiple monomers that form long chains. The process of binding these monomers in sequence to create long-chain molecules is called polymerization. While polymers can occur naturally, to form plastics, additives such as stabilizers, pigments, and fillers are incorporated during polymerization to enhance flexibility, durability, and thermal resistance. Plastics are categorized into two main groups: thermoplastics and thermosetting plastics (Crawford, 1998).

During World War II, plastic production became more widespread and continued growing rapidly in the following years, making the 1950s a defining period for the mass production of plastics (Streit-Bianchi et al., 2020). In 1950, the plastic production rate was 1,5 Mt globally, and this number reached 230 Mt in 2009 with a growth of 9% per year, and it is estimated that by 2050, 1.1 billion tons of plastic will be dumped in the environment (Rana, 2025). Of course, it was inevitable that an increase in plastic production would occur in recent years especially with new developments making plastic material more versatile. However, the rapid growth in plastic production also resulted in alarming plastic pollution due to mismanagement of plastic residues (Pottinger et al., 2024). Especially with the breakout of the COVID-19 pandemic, plastic pollution has been increased (Rivas et al., 2022). Changes in daily habits, such as preferring single use plastics to prevent any infection, usage of personal protective equipment (i.e., masks, gloves), have a significant contribution to the plastic pollution (Winton et al., 2022).

Furthermore, lockdown during the pandemic has increased home delivery food and online shopping, which resulted in increased plastic packaging wastes (Shams et al., 2021). Overall, since the 1950s, plastic production has only increased exponentially over the years, and according to the latest report of Plastic Europe, 413,8 Mt of plastic was produced in 2023. Figure 2.1. shows the amount of plastic produced worldwide from 1950 to 2021. Due to the mismanagement of plastic waste, pollution has reached an alarming level.

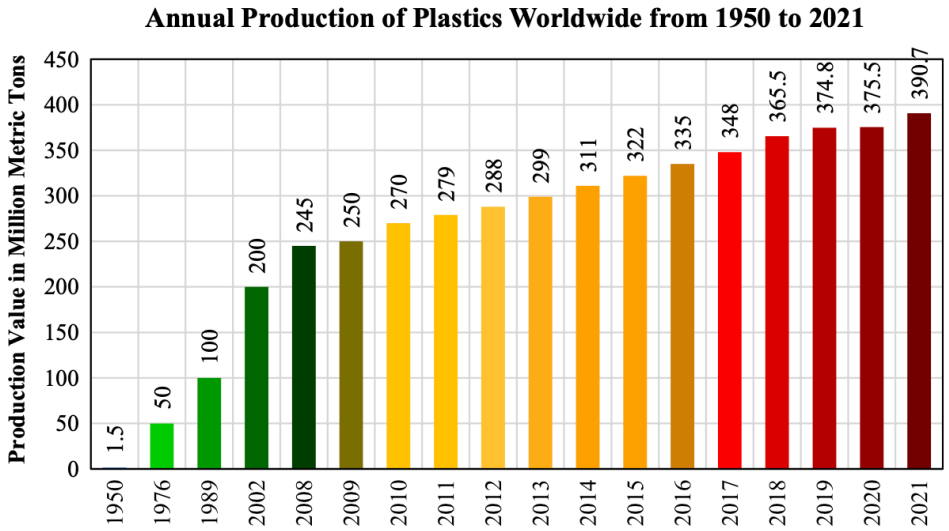


Figure 2.1 Worldwide plastic production (Haque et al., 2023)

There are two main types of plastic, which are namely the thermoplastics and thermoset plastics. Thermoplastics are plastic polymers that can be heated and cooled down, and during the heating process, they melt, and during the cooling process, they can be hardened again (Guney Yilmaz et al., 2024). They have non-crosslinked structures, and in addition, they are flexible, lightweight, and recyclable (Vaidya & Chawla, 2008). Their application areas are packaging, bottles, and household items. Polyethylene (PE), Polypropylene (PP), Polyvinylchloride (PVC), Polystyrene (PS), and Polyethylene terephthalate (PET) are examples of thermoplastics. Thermosets or thermosetting plastics are rigid and heat-resistant due to their cross-linked polymer chain form (Z. Wang et al., 2020). Once set, they cannot be reshaped. Excessive heat application can destroy the structure of thermosets, which is why they are not suitable for recycling. Their application areas are electrical components, cookware handles, and automotive parts (Ozturk et al., 2024). Polyurethane (PU), epoxy resins, phenolic resins, and melamine formaldehyde are examples of thermoset plastics.

2.2. Microplastics

Microplastics are defined as plastic particles smaller than 5 mm in size (Vardar et al., 2021). They vary in terms of polymer type, shape, size, and color. The most commonly encountered polymer types among microplastics include polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), polyethylene terephthalate (PET), and polyethylene (PE) (Ashrafy et al., 2023). Microplastics are generally categorized according to their shapes as fibers, fragments, films, foams, and pellets (Fiore et al., 2022). Their morphological and polymeric characteristics strongly influence their environmental fate and transport (Mutuku et al., 2024). Therefore, understanding these characteristics is important when interpreting their behavior in the environment.

In terms of origin, microplastics are divided into two categories: primary and secondary (Bostan et al., 2023). Primary microplastics are intentionally manufactured to be smaller than 5mm and are used in products such as pharmaceuticals, personal care products (toothpaste, shampoo, facial cleansers), and detergents (Lakhiar et al., 2024). Secondary microplastics, on the other hand, are formed through the degradation of large plastic items into smaller fragments. This degradation can occur through mechanical, chemical, and biological processes (Akdogan & Guven, 2019; Hu et al., 2019). Fibers released from washing synthetic clothing and particles from automobile tires are common examples of secondary microplastics.

Microplastics have been detected in marine and freshwater systems, terrestrial ecosystems, and the atmosphere across the world. Recent studies have shown the pervasive occurrence of microplastics in the Arctic marine environment; they have been found on the surface of the sea, even in the depths of the sediment (Tekman et al., 2020). Particle size and fragmentation processes play an important role in the dispersion and long-range transport of MPs (Onink et al., 2022). While human activities initiate microplastic formation and accumulation, environmental factors such as wind, water currents, and tides further facilitate their global spread (Shahul Hamid et al., 2018). An overview of these pathways is presented in Figure 2.2.

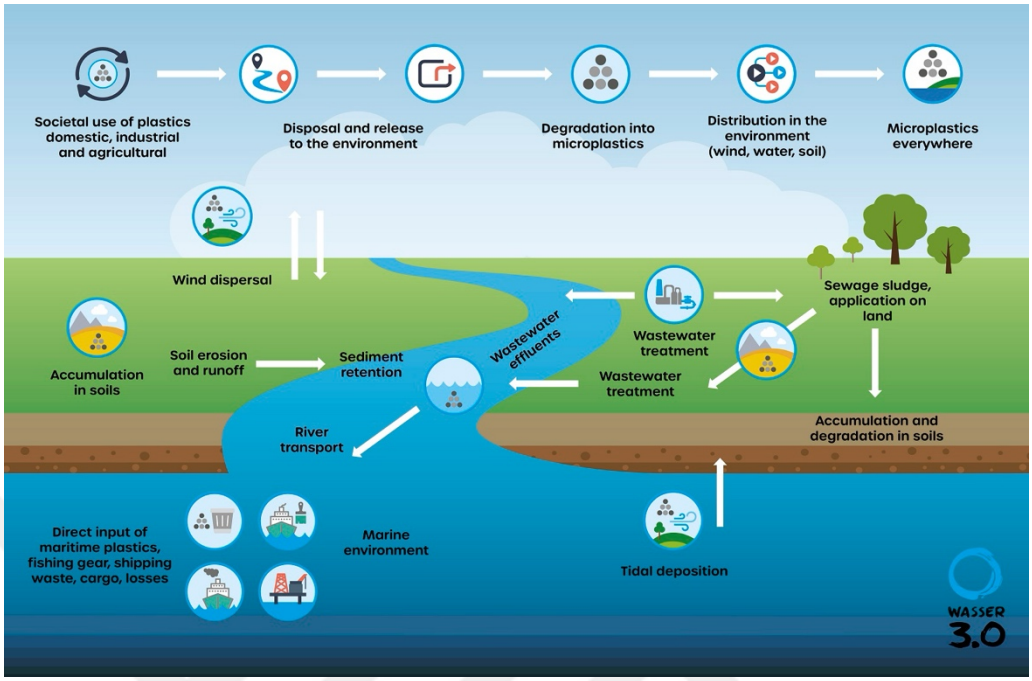


Figure 2.2. Pathways of microplastics generation and environmental dispersion (Wasser 3.0, n.d.).

Atmospheric microplastics emerge from several sources, including industrial emissions, tire wear, and urban activities (Y. Liu et al., 2025). In addition, dust from agricultural fields and sea spray generated by wave action have been identified as important pathways for the dispersion of atmospheric microplastics (Choudhury et al., 2023). Microplastics in the atmosphere exhibit mobility depending on their density and shape (Edo et al., 2023). In highly populated cities, transportation vehicles also contribute microplastic resuspension and dispersion in the air as well (Edo et al., 2023).

Indoor microplastics primarily arise from synthetic textiles and household items, with fibers being the dominant form (Amato-Lourenço et al., 2022). Some studies report that indoor microplastic concentrations are higher than outdoor levels (Amato-Lourenço et al., 2022; Z. Liu et al., 2022; Prata et al., 2020). Torres-Agullo et al. (2022) study reported microplastic concentrations reaching up to 17.3 ± 2.4 MPs/m³ in buses and 4.8 ± 1.6 MPs/m³ in houses, and the majority of them being PA and PES fibers, indicating the inhalation exposure risk from synthetic textiles. Atmospheric microplastics end up in soil or water via dry deposition (gravity) and wet deposition (rain, snow), with wet deposition being the dominant pathway (Kernchen et al., 2022).

The terrestrial environment is considered a major sink for microplastics (Rai et al., 2023; Zhao et al., 2022). Agricultural activities, such as sludge application, are regarded as a primary source of

microplastic contamination in the soil (de Souza Machado et al., 2018). Uncontrolled plastic waste also degrades into microplastics in soil (Rai et al., 2023). Tire wear is another significant source; Jan Kole (2017) estimated that 5.9 million tons of tire dust are generated from vehicle tires per year. Microplastics dispersion through the lower compartments of soil can happen via earthworms' movements (B. Xu et al., 2020). Heinze et al. (2021) showed that deep-burrowing earthworms can carry nano-plastics down into deeper soil layers through ingestion and excretion. Soil properties, climate, and hydrological events such as heavy rainfall also influence microplastic movement (Heinze et al., 2021).

Microplastics are also widespread in freshwater systems all over the globe. Urbanization and population density are major contributors to microplastic contamination in freshwater (Gutiérrez-Rial et al., 2024). WWTPs act as important point sources, releasing considerable quantities of MPs into freshwater systems via their effluents (Kumar et al., 2025). In addition, through abandoned ropes and nets, fishing activities cause MP pollution in lakes and rivers (Ding et al., 2021). Seasonal factors influence the distribution and migration of microplastics in freshwater ecosystems, for example, H. He et al. (2024) reported that microplastic abundance positively correlated with rainfall, and higher concentrations were observed during the rainy season.

In marine environments, WWTPs effluents act as important pathways for the release of synthetic fibers and residual microplastics, including those originating from personal care products (Saha & Saha, 2024). Additional inputs come from river discharge and land-based runoff (Li et al., 2023). Eventually, most plastics produced on land find their way into the ocean (de Souza Machado et al., 2018). Waves, tides, and wind facilitate further dispersion (Deng et al., 2023).

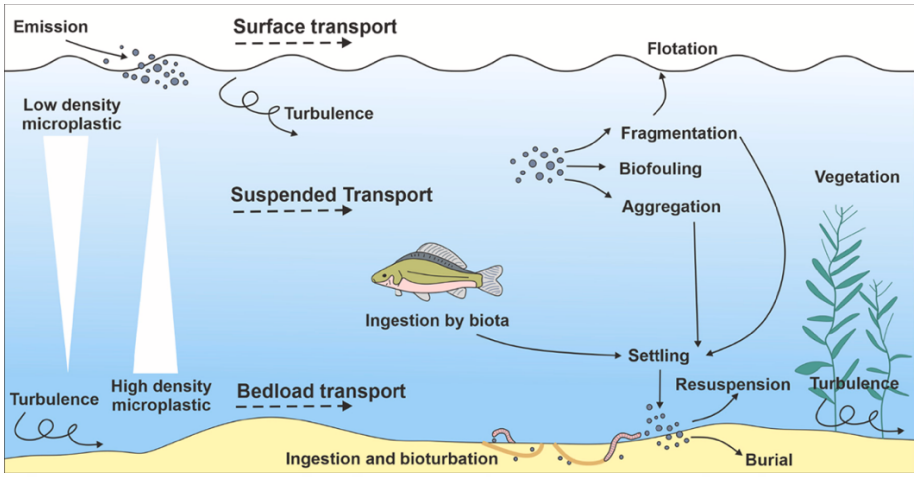


Figure 2.3. Pathways of microplastic transport and fate in aquatic environment (Waldschläger et al., 2022).

Once entered into the marine environment, microplastics undergo physical, chemical, and biological processes that determine their distribution (Bigdeli et al., 2022). An overview of these processes is illustrated in Figure 2.3. Low-density polymers float, whereas high-density polymers sink. Biofouling increases microplastic density over time, causing floating plastics to sink and accumulate in sediments (Waldschläger et al., 2022). Over time, environmental stressors including, sunlight, waves, tides, and turbulence, contribute to the fragmentation and degradation of MPs, thereby increasing their abundance in the marine environment (Sorasan et al., 2022). The top five polymers commonly found in the marine environment are listed as PE, PET, PP, PPA, and PS, with PPA, PS, and PET being the most prevalent across the five oceans. PE, PP, and PS were reported to be dominantly found at the surface of the water due to their low densities (Mutuku et al., 2024).

2.3. Microplastics' Effects on Health and Environment

Uncontrolled microplastic accumulation in receiving environments raises concerns for both ecosystem and human health due to microplastics' persistence and their potential to bioaccumulate in the tissues of living organisms. Moreover, microplastics have been associated with the transport of toxic chemicals and pathogenic microorganisms (S. He et al., 2022). In addition to these chemical and biological risks, MPs can also cause physical harm to organisms that ingest them, including choking and internal injuries (Zheng et al., 2023).

Across all compartments of the environment, living organisms are likely to encounter MPs. Ingestion of MPs has been reported as a potential cause of endocrine disruption, interfering with hepatic, renal, and gastrointestinal functions (Tang, 2025). Several studies report that ingestion of microplastics can cause reproductive and developmental toxicity (Tang, 2025). Experimental studies in animals further support these observations. For instance, Deng et al. (2017) exposed mice to microplastics with sizes between 5μm and 20μm for 28 days; at the end of the experiment, the exposure caused a decrease in adenosine triphosphate (ATP) levels as well as cholesterol levels, which was associated with changes in lipid metabolism. In addition, microplastics can disrupt seed germination and physically block the roots of plants (Tang, 2020). It is also known that microplastic can affect photosynthesis efficiency and algal growth rates (Tang, 2025). Pignattelli (2020) demonstrated that exposure of *Lepidium sativum* (garden cress) to polyethylene MPs for six days induced oxidative stress, reduced seed germination, and negatively affected biomass yield.

Low-density microplastics that cover the surface of the ocean prevent sunlight, thus adversely affecting the growth of photosynthetic plants and algae (Deng et al., 2023). These particles are also ingested by marine organisms, causing a false sense of satiety and consequently reducing nutrient intake. Moreover, microplastics are known to accumulate in various marine species, including mussels, shore crabs, and fish, where they can cause physiological and toxicological effects such as cellular dysfunction and bioaccumulation of hazardous chemical pollutants that may spread through the food chain. These effects may endanger human health through seafood consumption in addition to threatening marine biodiversity (Onyena et al., 2022).

Microplastics' ability to absorb organic material is an important concern in relation to their toxicity. Adsorption of organic matters such as pharmaceuticals and personal care products, where microplastics serve as carriers of these organic pollutants, can lead to mixed toxicity effects and potentially increase toxicity to organisms (H. Park & Park, 2021). Studies have demonstrated that plastics not only adsorb polycyclic aromatic hydrocarbons (PAHs) and persistent organic pollutants (POPs) but also carry them across the marine environment, promoting their bioavailability and easing their movement through the food web (Onyena et al., 2022). Additionally, leaching of additives such as phthalates and bisphenol A (BPA) further exacerbates their toxicity (Onyena et al., 2022). These additives can leach from microplastics during the environmental weathering process, releasing harmful chemicals such as nonylphenol with a half-life of several days in aquatic environments (Saud et al., 2023).

Moreover, microplastic surfaces carry not only chemical pollutants but also microorganisms, including potentially pathogenic species (Prata et al., 2020). Several studies have shown that microplastics can also carry antibiotic-resistant bacteria (ARB) and antibiotic-resistant genes (ARGs), which may facilitate their dispersion in the aquatic environment (Yang et al., 2020).

2.4. Microbial Colonization of Microplastics

Settling on the surface of plastic materials, microorganisms such as bacteria, fungi, and algae, form a region known as the "plastisphere" (Mishra et al., 2022). These biofilms represent structured microbial communities that allow interactions among different microorganisms. Biofilm formation on microplastic surfaces is a multistep process that begins with the formation of a conditioning film, where the microplastic becomes coated by a thin layer of naturally occurring organic and inorganic substances in the environment. This conditioning film alters the surface properties of the microplastic, making it easier for microorganisms to attach and initiate biofilm development. Microorganisms adhere to the surface using pili and flagella, and produce a sticky substance called EPS (extracellular polymeric substances) that helps form a stable biofilm (Rummel et al., 2017).

Microbial colonization and biofilm formation on microplastic surfaces are strongly influenced by both the characteristics of the substrate, such as polymer type, particle morphology, presence of additives and local factors including environment conditions and surrounding microbial community compositions. (Yang et al., 2020). The hydrophobic nature of plastics makes them suitable for microbial colonization, since bacterial cells tend to initiate hydrophobic interactions with plastic materials, facilitating microbial adhesion (Mishra et al., 2022). Polymer surface characteristics, such as surface texture and chemistry, play an important role in biofilm formation. Rougher surfaces promote biofilm development by providing more attachment sites, whereas smoother surfaces are less favorable due to limited attachment sites (Verma et al., 2024). The density of microplastics is another important property influencing biofilm formation, as it also affects the particle's behavior in the aquatic environment (Ayush et al., 2022).

For instance, denser polymers, such as polystyrene (PS) and polytetrafluoroethylene (PTFE), tend to sink, causing them to collide with surfaces and disrupt the biofilm stability, which potentially detaches existing biofilm layers. In contrast, lower-density polymers like polypropylene (PP) and polyethylene (PE) remain buoyant at the water surface, making them less prone to collisions and therefore more favorable for stable biofilm formation (Ayush et al., 2022). Another study by Ramsperger et al. (2020)

investigated the early-stage biofilm formation using PA, PET, and PVC type plastics in both freshwater and artificial seawater. Their results showed that beside polymer density, surface properties, and environmental context, altogether determine biofilm colonization patterns on microplastic. In a study by Jiang et al. (2023), biofilm growth on microplastics depends on the type of polymer. Among the polymers examined, PVC supported higher biofilm abundance, which was attributed to its surface characteristics, including roughness and chemical composition.

Furthermore, microplastic properties, hydrophobicity, roughness, and long half-life also benefit microorganism against severe environmental conditions; in other words, microplastics provide a steady surface for microorganisms (Zheng et al., 2023). As the biofilm matures, the microplastic surface starts the support a variety of complex microbial communities. The stability and protection that biofilms provide allow microorganisms, including potentially harmful ones, to persist under tough conditions.

In a study by Kong et al. (2024), the microbial community composition of bulk sludge samples containing microplastics in anaerobic sludge from WWTPs was investigated using 16S rRNA gene sequencing. The analyses revealed that predominant phyla across all samples were *Proteobacteria, Chloroflexi, Actinobacteria, Bacteroidetes, and Planctomycetes.* Specifically*, Proteobacteria* (36.6%–39.1%) and *Chloroflexi* (14.7%–40.5%) were among the most abundant, with variations depending on the sample sites. The study did not report differences between MP-associated and bulk sludge microbial taxa.

Hong et al. (2023) conducted an in-situ incubation study distributing PS microplastics to primary, secondary and tertiary WWTP stages to investigate microbial community on MPs. Using 16S rRNA gene amplicon sequencing, they analyzed microbial taxa on MP and in background waters, which were collected from real WWTPs, to analyze the general community. They found the MP-associated microbial community differed from the general community. While background waters were initially dominated by *Gammaproteobacteria*, the microplastic-associated communities showed increased abundances of *Bacteroidota*. Additionally, *Firmicutes* were notably increased on MP, particularly in the primary treatment stage, indicating MPs as carriers of diverse and resilient microbial assemblages.

In another in-situ experiment by Martínez-Campos et al. (2021), seven types of microplastic pellets were subjected to full-scale WWTP effluent water, and the formed biofilm was analyzed for bacterial communities and antibiotic resistance genes. Using Illumina MiSeq sequencing of 16S rRNA genes,

researchers observed significantly higher bacterial diversity on MPs compared to free-floating bacteria in the WWTP effluents. At the phylum level, the most abundant microbial taxa colonizing MPs and free-floating bacterial communities were similar and included *Proteobacteria, Bacteroidetes*, and *Actinobacteria*, while significant differences emerge at the family level. *Comamonadaceae, Rhodocyclaceae, Moraxellaceae, Hyphomicrobiaceae*, and *Rhodobacteraceae* were more abundant on MPs compared to free-floating bacteria.

Kruglova et al. (2022) investigated microbial taxa on MP through municipal wastewater treatment stages to assess bacterial diversity. They collected samples from influent, activated sludge, secondary and tertiary treatment, and effluent. They extracted MPs from samples using filtration and rinsed with saline solution, collected into Eppendorf tubes then subjected to the DNA extraction and analysis. Their 16S rRNA gene amplicon sequencing results showed that *Proteobacteria* were the most abundant phylum in MP biofilms. Notably, pathogenic bacterial groups such as *Neisseriaceae, Streptococcus, Pseudomonas, Lactobacillus*, and *Acinetobacter* persisted on MPs and passed through treatment, while free-living bacteria were primarily composed of *Firmicutes* (30%) and *Fusobacteria* (17%), followed by *Proteobacteria* (9%), while activated sludge (AS) free-living bacteria were dominated by *Acidobacteria* (up to 50%, mostly *Microtrichaceae* family) and *Proteobacteria* (up to 27%).

Galafassi et al. (2021) examined microbial taxa on MP particles in treated wastewater by using 16S rRNA gene amplicon sequencing to compare communities on MP and free-floating bacteria in wastewater before and after the disinfection step. They extracted MPs under a stereomicroscope and washed them with autoclaved MP-free water put them into DNA extraction tubes. Their result showed that MP-associated microbes differed significantly in richness, composition, and network structure from the free-floating bacterial community. Notably, the plastisphere was enriched in genera such as *Chryseobacterium*, which includes potentially pathogenic bacteria, while the free-floating community contained higher abundances of other potentially pathogenic taxa, *including Legionella, Aeromonas, Bacteroides, and Escherichia-Shigella*. In contrast to the similar studies, their result showed MPs can serve as carriers of potentially pathogenic bacteria; however, their contribution is relatively lower compared to the free-floating bacterial community.

Overall, while most studies have examined microplastic colonization under laboratory conditions, much less is known about the effect of WWTPs in the formation of biofilm on microplastics, even though WWTPs contain a high abundance of MPs and microbial communities.

2.5. RBC Reactor Working Principles

The Rotating Biological Contactor (RBC) is an attached-growth biological treatment system containing large rotating disks partially submerged in wastewater (Figure 2.4). These disks provide surface area for microorganisms to attach and develop biofilm where degradation of organic pollutants occur. This kind of system enables diverse microorganism growth, which is important for nutrient removal. Disks are rotating through horizontal shafts and are exposed to wastewater and air; thus, necessary O_2 is provided for microorganism growth. In time, biofilm gets thicker as the disks rotate and microorganisms break down organic matter in the wastewater. After pre-treatment, wastewater flows into the tank where disks are placed and biologically treated. Treated water goes into the effluent tanks and finally ends up in the receiving environment.

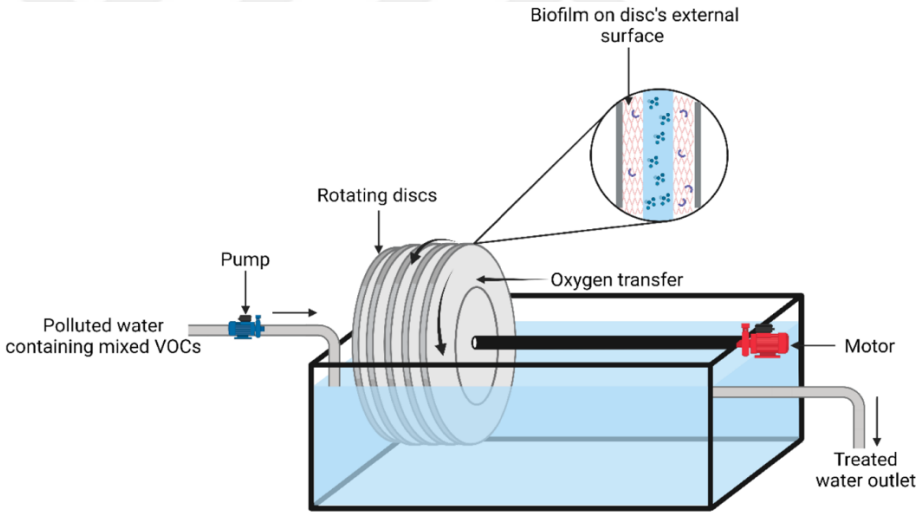


Figure 2.4. RBC unit schema (Baskaran et al., 2025).

In RBC units both aerobic and anaerobic degradation processes occur thanks to multilayered biofilm formed on the surface of rotating disks (Baskaran et al., 2025). Biofilm development stages include attachment of free-floating bacteria to the surface, aggregation of bacterial cells, growing and dividing of bacteria for biofilm creation, and matured biofilm formation (Waqas et al., 2021). After biofilm maturation, some cells detach and revert to planktonic form, allowing them to form new biofilms on new surfaces (Irfan et al., 2022). In time, biofilm gets thick due to growth and accumulation of microorganisms on the support surface (Coello et al., 2010; Hiras et al., 2004). Organic matter removal occurs mainly at the aerobic surface layer of the biofilm, while denitrification can take place in the deeper layers where oxygen concentration is limited (Waqas et al., 2021). Moreover, nitrification predominantly

occurs within the biofilm where sufficient oxygen is available to support the activity of ammonia-oxidizing bacteria, typically in intermediate layers between the aerobic surface and anoxic depths (Hassard et al., 2015).

To maximize the biological activity of the biofilm and ensuring efficient organic matter and nutrient removal from wastewater, it is very important to optimize operational conditions (Waqas et al., 2023). For example, with higher disk rotational speed, shear force, and fluid-induced mechanical stress increasing on the biofilm, it adversely affects the thickness of the biofilm (Hassard et al., 2015). Rotational speed is also crucial for dissolved oxygen (DO) level in the reactor (Irfan et al., 2022). Moreover, HRT (Hydraulic Retention Time) influences biofilm performance since it is directly related to the "contact time" between microorganisms and the wastewater (Irfan et al., 2022).

Having low operation cost, requiring minimal energy inputs while having high treatment efficiencies are some of the advantages of RBC systems (Waqas et al., 2023). Additionally, they have relatively simple designs and simple operational conditions, which make them attractive options (Huang et al., 2024).

2.6. Presence and Behaviors of Microplastics in WWTP

Wastewater treatment plants (WWTPs) are considered one of the most significant pathways through which microplastics (MPs) enter the environment (Ali et al., 2021; Raju et al., 2020; Sol et al., 2020; Vardar et al., 2021; Wu et al., 2021). Understanding the occurrence and fate of microplastics in WWTPs is therefore crucial to assess their environmental release. The most frequently detected MP types in WWTP influents are microfibers and plastic fragments, both of which mainly originate from secondary sources such as textile degradation and household activities (Sun et al., 2019), as well as microbeads derived from personal care products (Raju et al., 2018; Sun et al., 2019).

Bao et al. (2022) studied a tropical WWTP in China, evaluating microplastic presence through different treatment process units (primary treatment, secondary treatment, and sludge treatment stages) during dry and rainy seasons. The WWTP's removal rate was 94.09% and the most common microplastic shape was fibers in black color. They observed higher abundance and removal rate during the rainy season. In addition, secondary treatment was the main process for eliminating the majority of microplastics.

A nationwide study conducted in Korea by H. J. Park et al. (2020) investigated microplastic occurrence in 50 WWTPs with large treatment capacities above 50,000 m^3/day, employing A^2O or modified A^2O process. They found that overall removal efficiencies range from 98.7% to 99.99%. The most dominant MP shape was fragments, while PP was the most abundant polymer type. However, the authors emphasize that fiber shape microplastics and small size microplastic particles might have been underestimated due to the relatively large mesh size used in the study.

In their investigation of a conventional municipal WWTP in Thailand, Tadsuwan & Babel (2022) evaluated MP abundance and removal efficiency across different treatment stages, including a pilot-scale UF (Ultrafiltration) tertiary system. They found MP removal up to 86% in the conventional system, and with the addition of UF system, removal efficiency increased to 97%. The most dominant shape was fiber, consisting of 60% of all particles. Using spectroscopic analyses, they found PET as the major polymer type, particularly in fiber form.

In their study, Galafassi et al. (2021) investigated an urban wastewater treatment plant (WWTP) in Northern Italy, known as having the highest anthropogenic impact in Europe. The plant included primary screening, biological denitrification and oxidation, membrane filtration, and ozonation. Samples were collected from three different points: influent, pre-disinfection, and post-disinfection. They found that the WWTP efficiently retained over 98% of MPs. The majority of MPs were fragments, followed by fibers, while polyethylene (PE) and polypropylene (PP) were the most common polymers. Ozonation did not significantly affect MP abundance, size, type, or color, indicating that this step had a limited impact on MPs.

Franco et al. (2021) investigated MP contamination in two WWTPs, one urban and one industrial, located in Cádiz, Spain. Influent, effluent, and receiving waters were monitored in the study to assess MP abundance. Even though removal efficiencies exceeded 90%, discharged MPs were still substantial. Across all samples, fibers were identified as the dominant shape. Moreover, seventeen different polymer types were detected, including PVC, PE, ethylene-acrylic acid (EAA), and high-density polyethylene (HDPE), which were the most prevalent.

Correspondingly, studies from different regions of Türkiye, such as Sakarya (Bilgin et al., 2020), Mersin (Akarsu et al., 2020), and the Marmara Region (Vardar et al., 2021), have likewise reported microfibers as the dominant type of microplastic in WWTPs.

Can et al. (2023) investigated the properties and abundance of microplastics over a twelve-month period in a WWTP in Bursa, a province of Türkiye, whose effluent is discharged into the Marmara Sea. They found a high MP removal efficiency of 96% despite that they calculated approximately 74 million particles still discharged into the Marmara Sea daily. Fibers were the dominant shape, while PE, PP, and PET were primary polymer types.

Most existing studies have focused on suspended growth systems, whereas research on attached growth technologies remains scarce.

In a laboratory-scale study, Nur et al. (2022) observed MP removal efficiency using an attached-growth biofilm reactor (A_2O system) with raw wastewater from a WWTP in Neglasari district, Indonesia. The reactor achieved a high removal efficiency of 99.18%, reducing microplastic concentration from 810 MP/L in the influent to 10 MP/L in the effluent. Fibers and fragments were the dominant shape, while polymer characterization was mainly composed of polyester, PE, PP, PET, PA, and also cotton and wool were detected. The study noted that, besides sludge, biofilm was the sink for microplastics.

Le et al. (2023) evaluated MP abundance, morphology, and removal efficiencies across four WWTPs in Vietnam employing different treatment technologies, including conventional activated sludge, sequencing batch reactors, trickling filters (an attached-growth system), and aerated lagoons. Their results showed low MP removal in primary treatment, whereas high removal efficiencies (89%-99%) were achieved in secondary biological treatment, trickling filters, sequencing batch reactors, and activated sludge. The tertiary treatment unit, aerated lagoons, showed lower removal efficiency (60%). They found the most dominant shape as fiber in mostly blue and red colors, and major polymer types were PE (36%), PP (22%), and PES (21%), respectively.

Wang et al. (2020) examined the effectiveness of CWs in microplastics removal. The WWTP consisted of a primary settler, an RBC unit as a secondary treatment, and a horizontal subsurface CW as a tertiary treatment. They took samples from influent, effluent after secondary treatment, and the final effluent; the plant's overall removal efficiency was 98%. Removal efficiency for the combined primary

and secondary treatment was 87%. They found the most dominant shape was fibers, following fragments. Fragments and films were better removed from the plant (95%), while fibers removal efficiency was 81%.

Kılıç et al. (2023) examined three WWTPs in terms of MP composition, abundance, treatment, and load in Hatay province, Türkiye, whose effluents were discharged into the Orontes River. They found significant MP removal efficiency, with the highest achieved by the membrane bioreactor (99%), followed by the trickling filter (98%), and the activated sludge process (91%). Despite the high removal efficiencies, they calculated the daily MPs discharge into the Orontes River to be approximately 45 million particles. Fibers were the most dominant shape (94%), while PE, PET, and PA were identified as main polymer types.

Those consistent findings across various studies reveal that an important portion of microplastics is removed from the wastewater during treatment stages; however, because of the daily load of WWTPs all over the world, a huge part of microplastics continues to be discharged into the receiving bodies through effluent water of those plants, thus entering the environment.

3. MATERIALS AND METHODS

3.1. Sample Collection

Samples for microplastic analysis were collected from the Kilyos Wastewater Treatment Plant, located on the Black Sea coast of Istanbul Province. The plant serves a university campus, including its cafeteria and dormitory facilities. It has a capacity of 3,500 people and a flow rate of 620 m^3/day. At the time of sampling, the flow rate ranged between 35 and 53 m^3/day. The treatment plant includes a screen unit, a grease chamber, RBC tanks, and a sludge dewatering unit, as shown in Figure 3.1. The RBC tanks operate as attached-growth biological treatment units, where biofilm develops on rotating plastic media. Wastewater is equally distributed to four parallel RBC units. The disks rotate at a speed of 3–4 revolutions per minute (rpm), with approximately 40% of the disk surface submerged in wastewater. Each RBC tank has a rectangular geometry with dimensions of 2.35 m (width), 3.20 m (length), and a wastewater level of 1.25 m. Based on flow rates and tank dimensions, hydraulic retention time (HRT) was calculated to range between approximately 17 and 26 hours per RBC unit. Sampling points were selected as follows: (1) influent (after coarse screen), (2) inside the RBC tank reactor (TI), (3) biofilm (collected from the surface of the rotation disk), (4) final effluent, and (5) sludge. Samples for microplastic analysis were collected using a stainless-steel bucket and transferred into glass containers. Biofilm and sludge samples were directly collected into the glass jars, while effluent samples were taken directly from the outlet valve into glass containers. Samples were collected on three different dates in 2024: January 17, March 13, and May 29 to represent the winter, spring, and summer seasons. All samples were stored at +4^o C, and all pre-treatment steps prior to microplastic extraction were completed within one week, or at most two weeks.

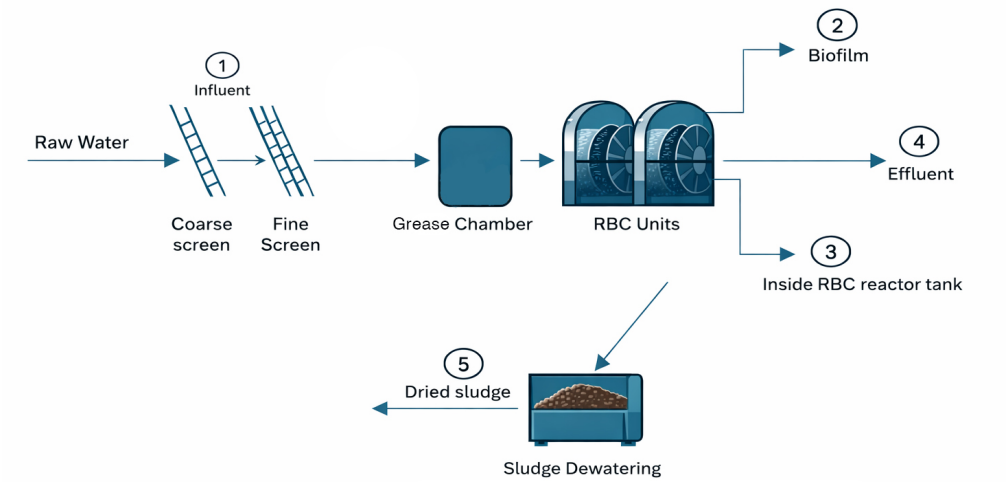


Figure 3.1 Flow chart of Kilyos WWTP.

Sample volumes were as follows: 5L for influent (IN), 1L for inside the RBC tank reactor (IT), and 15L for effluent (EFF). Biofilm (BF) and sludge (SLD) samples were collected directly into glass jars, without specific volume measurements.

To investigate the relationship between microplastics and microorganisms in an RBC-type WWTP, a total of 22 samples were collected into sterile Falcon tubes alongside sterilized glass containers. Samples were collected on three separate dates: 29.05.24, 5.11.24, and 13.02.25, respectively. The sampling conducted on 29.05.24 was associated with MP assessment in the WWTP, including abundance, distribution, and removal performance. In contrast, the samples collected in November (5.11.24) and February (13.02.25) were taken solely for microbial community composition examination.

For the analyses of microbial colonization on microplastic particles, sample volumes of 3L influent, 6L inside the RBC tank reactor, 9L effluent were collected into sterile glass jars. Additional samples from each point were collected in sterile Falcon tubes for total community analyses. Sludge and biofilm samples intended for microbial analysis were collected in 250 mL sterile Falcon tubes only.

3.2. Pre-Treatment Step

Wastewater contains a high amount of organic matter, which can interfere with microplastic extraction procedures. The presence of organic material in the samples complicates the detection of microplastics, making pre-treatment steps essential, particularly for samples with a high organic load.

The visual appearance of the samples indicates their organic load; darker samples generally contained more organic material, whereas light colored samples suggested less organic material (Vardar et al., 2021). Therefore, depending on the organic matter level appropriate treatment needs to be applied to effectively extract microplastic particles.

For microplastic analyses, all samples were sieved through steel mesh screens. Influent samples were sieved through 1mm, 500 µm, 250 µm, 125 µm, 63 µm, and 25 µm sizes. Effluent samples were sieved from 500 µm to 25 µm sizes, and samples from the RBC tank were sieved from 250 µm to 25 µm. Material retained on each sieve was rinsed into separate glass beakers using deionized water, and the tops of the beakers were covered with aluminum foil to prevent any contamination.

After all the retained material was transferred into beakers, the hydrogen peroxide (H_2O_2) method was applied, based on the procedure described by Vardar et al. (2021). A 30% H_2O_2 solution was added to the samples in small volumes (2 ml) and magnetically stirred. H_2O_2 was added incrementally over several days until no visible reaction remained, not exceeding a total digestion time of seven days. When necessary, especially for samples with higher organic content, the Fenton reagent was added to accelerate the reaction. The samples were heated up for 15 to 20 minutes while magnetic stirring. Approximately 3 grams of biofilm and sludge samples were directly subjected to H_2O_2 digestion. Once the digestion steps were complete, all samples were vacuum filtered on PVDF membrane filters (Durapore, 5 µm pore size, 47 mm diameter, Merck Millipore).

As a precaution against laboratory contamination, two petri dishes filled with deionized water (DI) were placed on benches while laboratory studies continue as suggested by Vardar et al (2021), to observe contamination. Laboratory coats made of cotton material were worn at all times. All laboratory equipment was rinsed with deionized water before use.

3.3. Visual Examination and Microplastic Extraction

The Zeiss Axio Zoom Stereo Microscope was used to examine the samples. Suspected microplastic particles were separated with micro tweezers under magnifications ranging from 20X to 80X, with most of the particles observed at 40X. Particles were transferred into clean petri dishes containing a glass microfiber filter (Whatman, 47 mm diameter).

Size measurements of the isolated particles were performed using the ImageJ software, and all data were recorded accordingly. Photographs of the samples were taken using Axicam506c, and the color and shape distributions of the separated particles were documented.

3.4. Microbial Analyses and DNA Extraction

To characterize the microbial community structure across the treatment system, bulk samples representing each operational unit (influent, effluent, biofilm, sludge, and the RBC tank) were first collected in sterile Falcon tubes. DNA extraction was conducted directly from these samples using the Zymo Quick-DNA Fecal/Soil Microbe Miniprep Kit.

In parallel, to examine microbial communities associated with microplastic particles, samples collected in sterile glass jars were vacuum filtered without any pretreatment, and suspected particles were separated into bead tubes and DNA extraction was performed using the same Quick-DNA Fecal/Soil Microbe Miniprep Kit to enable direct comparison with the bulk community samples.

All DNA extracts were subsequently stored at –20 °C. DNA concentrations were quantified using a Qubit fluorometer prior to PCR amplification.

3.5. PCR and DNA Sequencing

Extracted DNAs were subjected to 16S rRNA gene amplification using the NEXFLEX 16S V4 Amplicon-Seq Kit 2.0, which targets the bacterial 16S rRNA genes fourth hypervariable region (V4). PCR reactions were prepared following the kit protocol, using the provided supplied master mix and primer set. The protocol includes an initial amplification step followed by barcoding and corresponding cleanup procedures. Amplification was carried out on Bio-Rad T100 instrument under the cycling conditions recommended by the manufacturer. The resulting PCR products were examined by agarose gel electrophoresis to verify successful amplification and to confirm that the amplicon size matched the expected V4 region fragment. Individual amplicon libraries were quantified and adjusted to same molar concentration. The normalized libraries were then combined in equal volumes to prepare final pooled library for sequencing.

The sequencing procedure was performed using the NovaSeq 6000 (Illumina, USA) next-generation sequencing platform and the NovaSeq 6000 S4 Reagent Kit (Illumina, USA) with paired-end (2×150 bp) reads, following the manufacturer's instructions. The per-sample read counts were obtained.

After sequencing, the FASTQC tool was used for quality control of the obtained read data. Based on the quality control results, the data volume, read qualities, GC distributions, k-mer distributions, and potential adapter contaminations of each sample were examined. During the sequencing workflow, to prevent low-quality base calls and potential adapter-index contaminations in the raw reads from causing deviations in further analysis steps, the reads were trimmed. For trimming based on quality scores, the Trimmomatic tool (v0.39) was used (Bolger et al., 2014). In this step, low-quality bases at the read ends, possible adapter contaminants, and chimeric sequences were trimmed using the Genomes Online Database (GOLD) as a reference and the Trimmomatic tool.

For taxonomic profiling, the reads were aligned to target organisms using the Kraken2 tool with the RefSeq (Standard-Full) database (February 2022) (Quast et al., 2013; Wood et al., 2019). After alignment, OTU groups in each sample were identified. The vegan package in R was used to calculate diversity indices (Oksanen et al., 2017).

3.6. RAMAN Analyses

Renishaw Qontor Raman Spectroscopy was used for identifying the structure and molecular imaging of extracted microplastic particles. Analyses were performed using a 785 nm edge laser or a 532 nm laser using a 50x objective lens. Instrument settings were optimized individually for each particle to avoid thermal damage while achieving sufficient signal quality.

In general, for extended scans, laser power was typically set between 0.01-1% depending on the particles' thermal stability. The number of accumulations was set to 1-2, while 20-45 s exposure times were set. When higher spectral resolution was needed, static scans were performed. In static scanning, higher laser powers (5-10%) were applied, as the shorter measurement windows reduce the risk of thermal damage. The number of accumulations was set to 2-3, and 20-45 s exposure times were set as in extended scans. All the settings were made to improve the signal-to-noise ratio. To identify each particle material type, all spectra backgrounds were corrected and compared to reference polymer libraries.

4. RESULTS AND DISCUSSION

In three different seasons, samples were collected from Kilyos WWTP, and in total, 6,160 suspected microplastic particles were extracted from all the samples. Figure 4.1 presents examples of zoomed-in photos of some of those extracted particles.

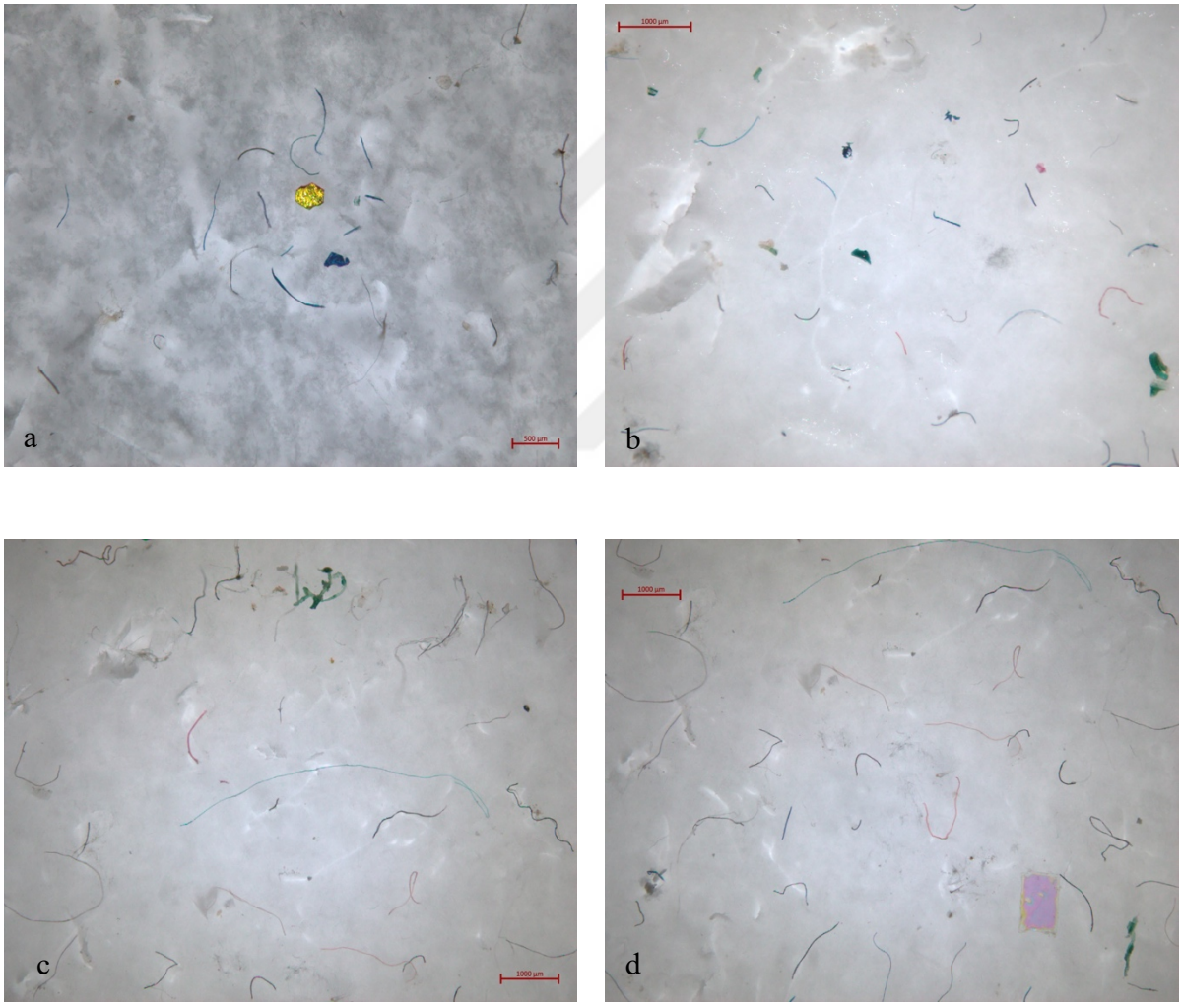


Figure 4.1 Particles separated from influent samples (a from 14.03.24 influent, b-c-d from 17.01.24 influent).

In the winter season, a total of 2,197 particles were extracted. The influent exhibited the highest number with 1,754 particles, followed by sludge (341 particles), effluent (64 particles), and biofilm (38 particles).

Fibers were the dominant shape across all sampling points, comprising 94% of influent particles and 86% of effluent particles. In the influent, fiber colors were largely black (706) and blue (501), with 189 red and 179 gray fibers. Effluent fibers were primarily black (25) and transparent (15), whereas biofilm fibers were distributed mainly among black (10) and blue (11). Sludge samples were dominated by black (165) and blue (90) fibers.

Soft fragments occurred in smaller numbers, with 86 particles in influent, 8 particles in effluent, and 3 particles in sludge, mostly green, blue, and red. Hard fragments were relatively rare, with 18 particles in influent, primarily green. Additionally, a single yellow glitter particle was observed in influent, while one shiny black hard fragment was detected in effluent.

In the spring season, 1,453 particles were extracted. The influent contained 727 particles, followed by sludge (438), effluent (181), biofilm (54), and the RBC tank (53) particles.

Fibers again dominated all the matrices, comprising 88% of influent and effluent particles, 89% of biofilm, and 95% of sludge samples. In influent, fibers were primarily black (218) and blue (163), followed by red (113) and transparent (71). Effluent fibers displayed a similar trend, with black (47) and blue (51) as the most frequent colors, along with transparent (35). The RBC tank contained mostly black (14), blue (11), and transparent (18) fibers. Biofilm fibers were dominated by black (31), whereas sludge fibers were mostly black (254) and blue (68).

Fragments are classified as hard and soft according to their physical properties. Soft fragments were most abundant in the influent with 73 particles, dominated by green and blue colors. Hard fragments were relatively rare but included red and blue particles in influent, and one shiny black hard fragment was recorded in effluent.

In the summer season, a total of 2,510 particles were extracted across all sample points. The influent accounted for the highest particle abundance (1,597 particles), followed by sludge (456 particles), effluent (209 particles), the RBC tank (202 particles), and biofilm (46 particles).

Fibers were the dominant shape in all matrices, representing 91% of influent, 83% of effluent, 94% of sludge, and 98% of biofilm particles. Hard and soft fragments together represent 8% of the total. In influent samples, fiber colors were primarily black (532) and blue (428), followed by red (190), gray

(171), and transparent (54). Effluent fibers showed a similar pattern, with black and blue being the most frequent colors (56 each), while transparent (35) and red (13) were also present. Sludge samples were likewise dominated by black (227) and blue (94) fibers, whereas RBC tank fibers were more evenly distributed among black (68), blue (21), transparent (21), and red (20). Biofilm samples, though few in total, were mostly black (16) and transparent (18).

Soft fragments were most abundant in the influent (132 particles), primarily blue and red, while hard fragments were rare, accounting for only 1% of all particles extracted.

Overall, particle abundance varied seasonally, with the highest counts in the summer (2,510), followed by winter (2,197) and spring (1,453), indicating seasonal variation. Fibers were consistently the dominant shape across all seasons and sampling points, accounting for 92% of all particles. This is consistent with previous studies that also reported fibers as the prevailing microplastic type in wastewater treatment plants. Soft and hard fragments were detected in relatively low numbers, with slightly higher proportions in influent and effluent compared to sludge and biofilm. Additionally, one glitter and one film particle were detected in separate seasons. Unlike some other studies, no pellets or beads were detected in this investigation.

Comparisons with the literature reveal both similarities and regional differences. A study in Cadiz, Spain, also found fibers as the most abundant type, followed by fragments and flakes (Franco et al., 2021). Similarly, Lares et al. (2018) reported fibers as dominant, with fragments and spherical particles also present. In Türkiye, Vardar et al (2021) observed fibers as the predominant type across all matrices, with fragments as the second most common, while pellets and glitters were also detected. Gündoğdu (2018) reported contrasting patterns between two WWTPs in Adana: in Seyhan WWTP effluents, fibers, films, and fragments were found in similar amounts, whereas fibers dominated in Yüreğir WWTP effluents. In South Korea, 50 municipal WWTPs were monitored, and fragments were detected as the dominant shape in influent (68.2%) and effluent (82.3%), followed by fibers as the second dominant shape (H. J. Park et al., 2020). Similarly, (Yuan et al., 2020). examined two WWTPs in China and reported that, in both WWTPs, fragments and granules were the dominant shapes, while films and fibers were comparatively less frequent.

4.1. Microplastic Concentrations and Removal Efficiency in Wastewater Samples

MP particle concentrations exhibited in Table *4.1* indicate that winter and summer seasons exhibited higher MP loads, while spring had considerably lower levels. The highest influent concentration was recorded in winter (350.8 particles/L), followed by summer (319.4 particles/L). Despite these seasonal differences in influent loads, effluent concentrations remained consistently low across all sampling periods (12-14 particles/L), demonstrating stable removal performance of the system.

Table 4.1. Particles per liter for influent and effluent points for each season.

Season	**Influent (particles/L)**	**Effluent (particles/L)**	**Removal efficiency (%)**
Winter	350.8	12.8	96%
Spring	145.4	12.06	92%
Summer	319.4	13.93	96%

The WWTP investigated in this study is a closed system, meaning that precipitation does not affect the flow rate of the plant. Therefore, the seasonal variations observed in microplastic loads are likely related to behavioral changes among the students and staff on campus, which are influenced by the academic schedule. For example, the winter sampling was conducted during the final and make-up exam period. During this period, dormitories are expected to be more crowded, since student presence typically increases around exam times. Similarly, summer sampling took place during the final exams. In contrast, the spring sampling occurred during the regular class period, when student presence is generally more dispersed. Consequently, the observed seasonal difference in influent MP loads does reflect fluctuations in human activity patterns on campus.

Table 4.2. Stepwise removal efficiencies of all particles.

Season	Influent (particles/L)	TI (particles/L)	Effluent (particles/L)	Influent to TI removal (%)	TI to Effluent removal (%)	Influent to Effluent removal (%)
Winter	350.8	-	12.8	-	-	96.4
Spring	145.4	53	12.067	63.6	77.27	91.69
Summer	319.4	67.333	13.933	78.96	79.3	95.64

Stepwise removal efficiencies, as summarized in Table 4.2, indicate that the RBC system consistently reduces microplastic concentrations between influent and effluent across all seasons. During spring, 63.6% of MPs were removed between influent and TI, and an additional 77% were removed between the TI and effluent, resulting in an overall influent to effluent removal of 91.7%. In summer, 78.9% removal occurred between influent and TI, followed by 79% removal between TI and effluent, yielding an overall removal of 95.6%. In winter, only influent to effluent removal was calculated, which was 96.4%, consistent with the strong overall performance of the system.

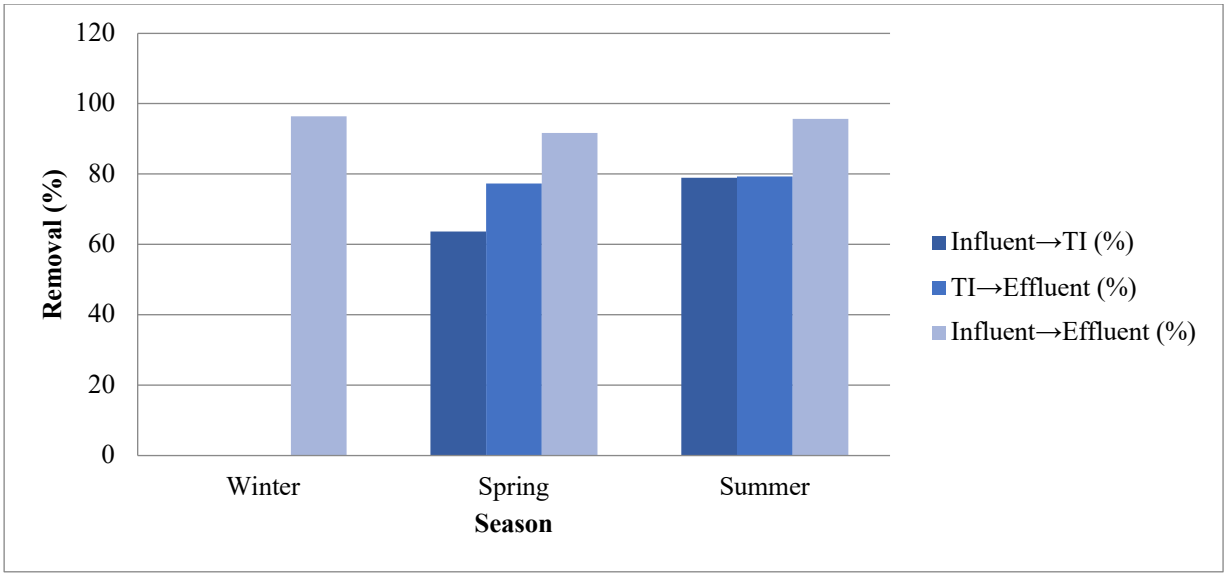


Figure 4.2. Seasonal comparison of stepwise microplastic removal in the RBC system.

Table 4.3. Stepwise removal efficiencies of fiber shape.

Season	Influent (fiber/L)	TI (fiber/L)	Effluent (fiber/L)	Influent to TI removal (%)	TI to Effluent removal (%)	Influent to Effluent removal (%)
Winter	329.8	-	11	-	-	96.67
Spring	127.4	51	10.67	59.97	79.01	91.63
Summer	291.6	55	11.6	81.13	78.91	96.02

Table 4.4. Stepwise removal efficiencies of fragment shape.

Season	Influent (fragment/L)	TI (fragment/L)	Effluent (fragment/L)	Influent to TI removal (%)	TI to Effluent removal (%)	Influent to Effluent removal (%)
Winter	20.8	-	1.8	-	-	91.35
Spring	18	2	1.4	88.89	30	92.22
Summer	27.8	12.33	2.33	55.66	81.07	91.61

Shape-specific removal trends are shown in Table 4.3 for fibers and Table 4.4 for fragments, respectively. Fibers consistently dominated the influent fraction across all seasons, and their removal efficiencies ranged between 92-97%, slightly higher than those for fragments (91-92%). This suggests that fibrous MPs are more effectively retained by the RBC process, likely due to their long structure being more easily trapped within the biofilm or sludge.

Table 4.5. Microplastic concentrations in sludge and biofilm.

Season	Sample	Sample amount (g)	Total particles	Particles/g
Winter	Biofilm	3.15	38	12.06
Winter	Sludge	3.35	341	101.79
Spring	Biofilm	3.39	54	15.93
Spring	Sludge	3.4	438	128.82
Summer	Biofilm	3.95	46	11.65
Summer	Sludge	3.45	456	132.17

Microplastic concentrations in sludge and biofilm are presented in Table 4.5 confirmed the accumulation of MPs within the system, with sludge containing 102-132 particles/g and biofilm containing 11-16 particles/g depending on the season. The consistently higher MP densities in the sludge indicate that this compartment acts as the primary MP sink, while biofilm also contributes to retention.

Overall, the RBC system demonstrated high efficiency in MP removal across all seasons, with fibers being more efficiently removed than fragments. In addition, sludge and biofilm samples confirmed the accumulation of MPs, especially in sludge particle densities ranging between 102-132 particles/g, indicating that these compartments act as MP sinks.

4.2. Distribution of Microplastic Size

In the winter season, influent samples were predominantly composed of particles in the 500–1000 µm range, followed by the 250–500 µm fraction. Fine particles (<100 µm) represented only 4% of the total. Effluent samples displayed a similar distribution, with the 500–1000 µm fraction being most abundant, followed by 250–500 µm, while fine particles accounted for only a minor proportion. Sludge samples were likewise dominated by the 500–1000 µm size range, with the 250–500 µm fraction as the second most abundant. Biofilm samples, in contrast, exhibited a relatively higher occurrence of fine particles (7.9%), although larger fractions still constituted the majority. These distribution patterns are summarized in Table 4.6, which present the count and the percentage of particles in each size class across all sample types during the winter season.

Table 4.6. Winter season particle size distributions.

Size Range (µm)	Influent (count, %)	Effluent (count, %)	Sludge (count, %)	Biofilm (count, %)
<100	71 (4.0%)	3 (4.7%)	1 (0.3%)	3 (7.9%)
100–150	100 (5.7%)	2 (3.1%)	6 (1.8%)	2 (5.3%)
150–250	231 (13.2%)	7 (10.9%)	28 (8.2%)	2 (5.3%)
250–500	442 (25.2%)	15 (23.4%)	92 (27.1%)	8 (21.1%)
500–1000	443 (25.2%)	17 (26.6%)	99 (29.1%)	5 (13.2%)
1000–2000	323 (18.4%)	14 (21.9%)	68 (20.0%)	11 (28.9%)
>2000	145 (8.3%)	6 (9.4%)	46 (13.5%)	7 (18.4%)
Total	**1755 (100%)**	**64 (100%)**	**340 (100%)**	**38 (100%)**

The plant exhibited uniform removal efficiency across all particle size classes during the winter. The highest removal was observed in the 100-150 µm fraction (98%), while the lowest removal occurred in the 500-1000 µm fraction (96.2%), but still being a substantial efficiency. Despite these slight variations, all size classes exceed 95% removal.

In spring, influent samples were enriched in the 250–1000 µm size range, a trend also observed in effluent and sludge. However, both effluent and sludge also contained a substantial proportion of larger particles (1000–2000 µm). Biofilm samples were dominated by larger particles, whereas the TI samples were mainly composed of 250–1000 µm particles, resembling the influent distribution. These distributions are summarized in Table 4.7, which presents the particle size classes and their relative proportions across all matrices during the spring season.

Table 4.7. Spring season particle size distributions.

Size Range (µm)	Influent (count, %)	Effluent (count, %)	Sludge (count, %)	Biofilm (count, %)	Inside RBC Tank (count, %)
<100	39 (5.4%)	8 (4.6%)	22 (5.0%)	9 (16.7%)	5 (9.4%)
100–150	41 (5.6%)	7 (4.0%)	27 (6.2%)	3 (5.6%)	3 (5.7%)
150–250	97 (13.4%)	19 (10.9%)	56 (12.8%)	2 (3.7%)	7 (13.2%)
250–500	185 (25.5%)	36 (20.6%)	93 (21.2%)	5 (9.3%)	12 (22.6%)
500–1000	213 (29.3%)	47 (26.9%)	107 (24.4%)	10 (18.5%)	16 (30.2%)
1000–2000	121 (16.7%)	47 (26.9%)	98 (22.4%)	10 (18.5%)	8 (15.1%)
>2000	30 (4.1%)	11 (6.3%)	35 (8.0%)	15 (27.8%)	2 (3.8%)
Total	**726 (100%)**	**175 (100%)**	**438 (100%)**	**54 (100%)**	**53 (100%)**

Size-based removal efficiencies during the spring season were notably lower than those observed in winter, ranging from 61% to 83% across the different size classes. The highest removal was observed in the 100-150 µm fraction (82.9%). The lowest removal occurred in the 1000-2000 µm and >2000 µm classes (61% and 63% respectively), indicating that larger particles were retained less effectively during this period.

In the summer season, influent MPs were dominated by the 250-500 µm fraction, followed by 500-1000 µm, while smaller fractions were less abundant. Effluent samples showed comparatively higher proportions of smaller fractions. Sludge and biofilm samples were enriched in larger MPs, particularly the 500–1000 µm fraction in sludge (30%) and the 1000–2000 µm fraction in biofilm (45.7%). These distributions are presented in the Table 4.8.

Size-based removal efficiencies during the summer season exhibit a wider variability compared to the winter and spring seasons. The highest removal occurred in the 150-250 µm (91%) and 250-500 µm (90.5%) fractions. In contrast, both the smallest (<100 µm) and largest (>2000 µm) size classes showed the lowest removal (66.0% and 66.7%, respectively).

Table 4.8. Summer season particle size distributions.

Size Range (µm)	Influent (count, %)	Effluent (count, %)	Sludge (count, %)	Biofilm (count, %)	Inside RBC Tank (count, %)
<100	94 (6.3%)	32 (15.3%)	10 (2.2%)	3 (6.5%)	57 (28.2%)
100–150	91 (6.1%)	18 (8.6%)	20 (4.4%)	2 (4.3%)	26 (12.9%)
150–250	201 (13.5%)	18 (8.6%)	50 (11.0%)	0 (0.0%)	33 (16.3%)
250–500	507 (34.2%)	48 (22.9%)	113 (24.8%)	2 (4.3%)	50 (24.8%)
500–1000	413 (27.8%)	54 (25.8%)	137 (30.0%)	8 (17.4%)	29 (14.4%)
1000–2000	157 (10.6%)	32 (15.3%)	87 (19.1%)	21 (45.7%)	5 (2.5%)
>2000	21 (1.4%)	7 (3.3%)	39 (8.6%)	10 (21.7%)	2 (1.0%)
Total	**1484 (100%)**	**209 (100%)**	**456 (100%)**	**46 (100%)**	**202 (100%)**

Overall, medium-sized particles (250–1000 µm) were the dominant fraction across influent, effluent, sludge, and TI samples in all seasons. Larger MPs (1000–2000 µm) were concentrated mainly in biofilm samples, while both biofilm and TI also contained relatively higher proportions of fine particles compared to other matrices. In addition, across all seasons, medium-sized particles exhibit higher removal efficiencies compared to both the smallest and largest size classes, showing that medium-sized particles were captured more effectively than other size classes.

One-way ANOVA was applied to assess seasonal differences in microplastic size distributions across influent, effluent, RBC tank reactor (TI), biofilm, and sludge samples. No statistically significant seasonal differences were observed ($p > 0.05$), indicating that size distribution patterns remained stable despite seasonal variations in particle abundance.

The size distribution patterns observed in influent and effluent samples are broadly consistent with those reported in previous studies. For instance, Lares et al. (2018) found that the dominant effluent size range was 0.5–1 mm. Yuan et al. (2020) reported shape-specific size differences, with fragment lengths typically within 500–1000 µm and average fiber lengths around 1100 µm. Similarly, Gündoğdu et al. (2018) showed that most fibers and films fell between 1–5 mm range, while fragments were frequently <100 µm. Vardar et al. (2021) also reported the majority of particles within the 500–1000 µm size range, followed by 1000–2000 µm, supporting the dominance of medium to large particles in WWTP matrices.

4.3. Polymer Type Classification of Microplastics

For the summer season, 76 particles were analyzed for polymer characterization, which constituted 3% of the isolated particles from the samples. These particles consisted of 14 isolated particles from sludge, 6 from effluent, 48 from influent, 6 from the tank reactor, and 2 from biofilm samples.

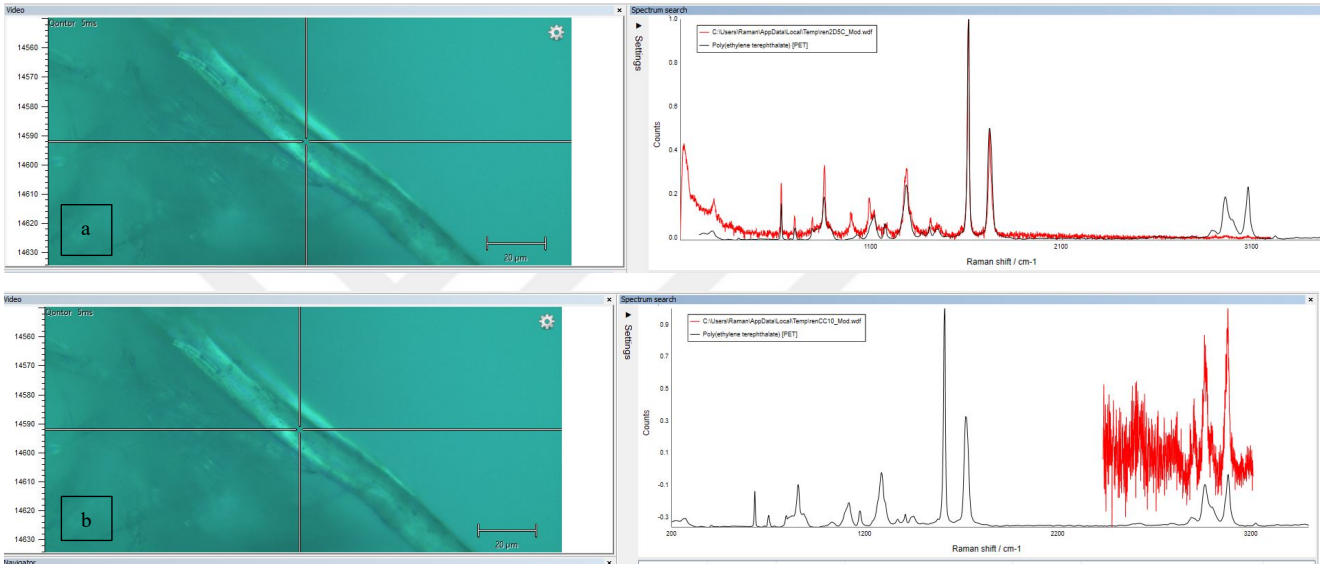


Figure 4.3 Extended (a) and static (b) Raman spectrums of a transparent fiber.

Among the 48 particles from influent, 45 fibers, 2 soft fragments, and 1 hard fragment constitute the particles. The fibers were dominantly black (13), 8 of them identified as PET, 3 nylon, 1 PVC, and 1 polyphenylsulfone (PPSU). Blue fibers (11) comprised 4 PET, 3 nylon, and 1 poly(4-vinylbiphenyl) (PVBP), with 2 spectra that could not be matched. Red fibers (9) included 4 PET, 2 nylon, 1 PVC, and 2 unidentified spectra. 4 out of 5 transparent fibers were identified as PET, and 1 unidentified spectrum. Other fiber colors included 1 yellow PVC fiber, 1 mix colored (red and transparent) PET fiber, 2 gray nylon fiber, and 1 purple PP fiber. Figure 4.3, Figure 4.4, and Figure 4.5 show Raman spectra of the analyzed particles together with their matching library references. The extended scan provides a broad overview of the spectrum, while static scan focuses on a narrower region. In those examples, the measured spectra match well with the library curves, confirming the polymer identification. Although, Figure 4.5 spectrum contains more noise, the main peaks are still visible and align with the reference data.

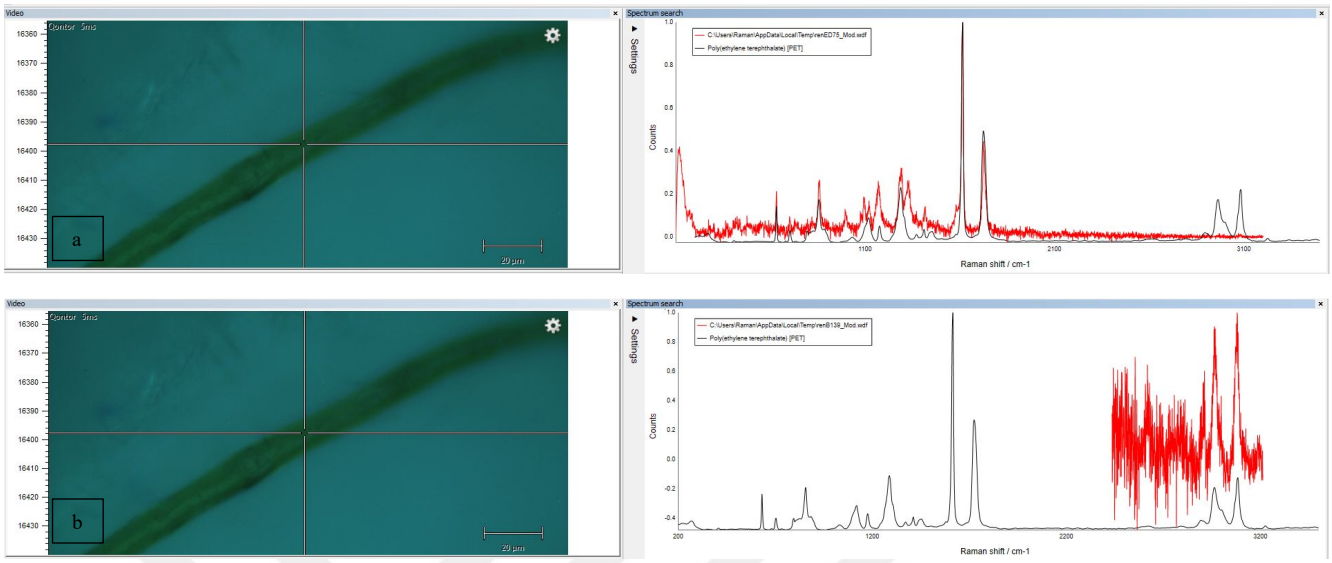


Figure 4.4. Extended (a) and static (b) Raman spectrums of a yellow fiber.

A total of 14 particles were selected from the sludge sample for characterization, including 3 transparent fibers, 3 black fibers, 2 blue fibers, and 2 red fibers. Among the black fibers, two were identified as PET, while no matching reference spectrum was obtained for the remaining fiber in the instrument library. Of the red fibers, one was identified as PVC and the other as PP. One blue fiber was identified as PVC, and 1 as PET. All 3 of the transparent fibers were identified as PET. Other particles include 1 green hard fragment (PP), 1 yellow fiber (PET), and 1 gray fiber (PET).

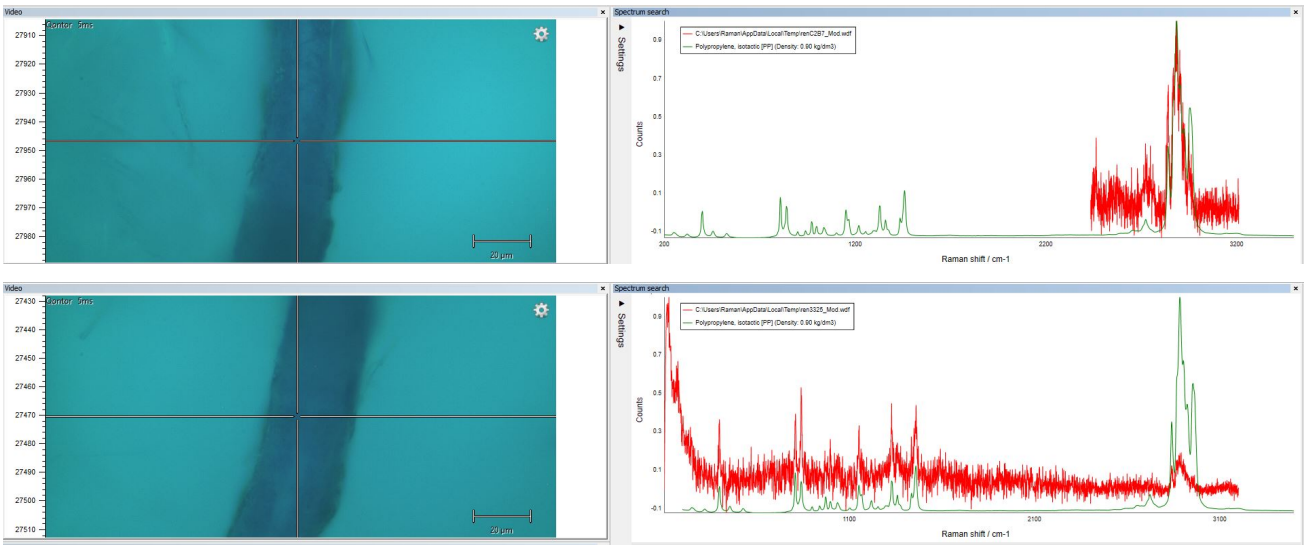


Figure 4.5. Extended (a) and static (b) Raman spectrums of a blue particle.

From the RBC reactor samples, 6 particles were analyzed. These included 2 transparent fibers (one PET, one nylon), 2 black fibers (one PET, one polyisophthalamide (PPA)), 1 blue fiber (PP), and 1 blue hard fragment (polyisophthalamide). 1 transparent fiber (PET) and 1 black fiber (nylon) were characterized from the biofilm sample.

6 particles identified from effluent samples, including 1 black fiber (PET), 2 transparent fibers also identified as PET, and 1 red soft fragment (nylon). A meaningful spectrum could not be obtained from a blue fiber. The spectrum of a blue soft fragment did not match any reference in the instrument library.

Table 4.9. Polymer distribution in wastewater treatment plant samples during summer season.

Sample type	**Total particles (n)**	**Identified (n)**	**Unidentified (n)**	**Identified (%)**	**Unidentified (%)**	**Dominant form**	**Polymers identified**
Influent	48	40	8	83.3	16.7	Fibers (45), Soft fragments (2), Hard fragment (1)	PET (17), Nylon (10), PVC (3), Polyphenylsulfone (1), Poly(4-vinylbiphenyl) (1), PP (1)
Sludge	14	12	2	85.7	14.3	Fibers (12), Hard fragment (2)	PET (6), PP (2), PVC (1)
Biofilm	2	2	0	100	0	Fibers (2)	PET (1), Nylon (1)
RBC reactor	6	6	0	100	0	Fibers (5), Hard fragment (1)	PET (2), Nylon (1), PP (1), Polyisophthalamide (2)
Effluent	6	4	2	66.7	33.3	Fibers (4), Soft fragments (2)	PET (3), Nylon (1)

Table 4.9 summarizes the polymer characterization results for all sample types in summer season. Influent samples exhibited the highest particle diversity with 48 particles analyzed, of which 83.3% were successfully identified. PET and nylon were the dominant polymers, accompanied by PVC, polyphenylsulfone (PPSU), poly(4-vinylbiphenyl) (PVBP), and PP, even though they were in small amounts. 85.7% of the sludge sample particles were successfully identified with PET and PP being the most dominant type. Biofilm and RBC reactor samples demonstrated a 100% identification success rate, though the total number of analyzed particles was relatively low. Effluent samples exhibited the lowest identification rate (66.7%), with PET and nylon as the primary detected polymers.

PET is widely used in the textile industry in fiber form and is also used heavily in the packaging industry (Oda & Wlodawer, 2024). PVC has broad industry applications, particularly in construction materials such as sewage and drainage pipes, as well as in textiles, electronics, and packaging (Arun & Meena, 2024). PP is commonly used in textiles, packaging, and molded plastic products, including bottles, containers, and household items (Maddah, 2016). Nylons are also used in the textile industry in fiber forms, in addition to their applications in mechanical parts (Arioli et al., 2024). Since most particles identified as PET, nylon, PP, and PVC were fibers, these polymers are likely textile-derived microplastics, most of which may have originated from washing machine effluents. Given that the plant operates as a closed system, PPA can be attributed to abrasion of internal equipment or infrastructure, as PPA-based components are considered to be used in electrical water pumps and related systems (Gonzalez de Gortari et al., 2022). Polyphenylsulfone (PPSU), which is used in high-temperature applications such as laboratory autoclave equipment, may have originated from laboratory activities within the campus. Unmatched graphs and inconclusive spectrum results may have been caused by organic residues remaining on particle surfaces that interfered with clear Raman signal acquisition.

To provide an overall perspective on analytical performance, Figure 4.6 presents the combined identification success rate across all samples. The pie chart clearly illustrates that the majority of analyzed particles (84%) were successfully characterized, while a smaller fraction remained unidentified.

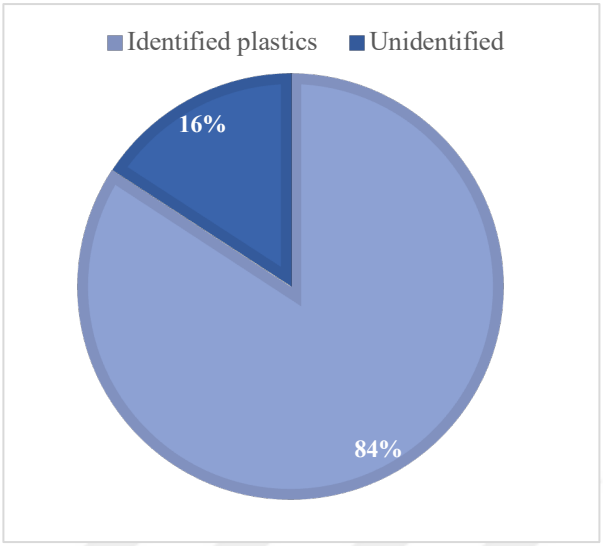


Figure 4.6. Summary of identified and unidentified particles across all sample types in summer season.

Overall, in the summer season, PET and nylon emerge as the most prevalent polymers across all sampling points, which differs from many studies reported in the literature. For instance, Yuan et al. (2020) found PE and PP to be the dominant polymer types in both WWTPs analyzed, alongside smaller proportions of PS, phenolic resin, and PET. Similarly, Gündoğdu et al. (2018) reported polyester (50.8%), PE (29.2%), and PP (13.8%) as the main polymers in Seyhan WWTP, while Yüreğir WWTP showed the same trend, with polyester being the most abundant, followed by PE and PP. In another study by H. J. Park et al. (2020), PP, PE, and PET were reported as the dominant polymers in both influent and effluent samples of examined WWTPs.

4.4. Bacterial Community Analyses

November and February sampling events focused on characterizing both microplastic associated microbial communities and general microbial communities across the treatment units. Among these, May 2024 (May24) sampling served a dual purpose: in addition to providing MP abundance and removal data, it was also used to establish a baseline microbial community for comparison with the November 2024 (Nov24) and February 2025 (Feb25) datasets.

Taxonomic classification of sequencing reads was performed using Kraken2, and the resulting taxonomic profiles were visualized in MultiQC as bar plots showing the dominant taxa across the domain and species range. Microbial community comparisons were carried out across different grouping

schemes. First, general communities from different seasons were compared to assess whether the overall microbial structure remained consistent and how seasonal changes affected the general community. Second, within each season, general communities were compared with microplastic-related communities to evaluate whether microplastics hosted distinct microbial assemblages.

DNA extractions generally produced adequate amounts of DNA across most samples, although there were a few clear exceptions. The MP samples collected in February 2025 (MP_inf_Feb25 and MP_sld_Feb25) yielded extremely low concentrations, around 0.02–0.09 ng/µL. The total number of sequencing reads obtained for each sample, along with the proportions of taxonomically classified and unclassified reads, is presented in Figure 4.7 Most samples produced a relatively high share of classified reads; however, a noticeable drop was observed in the Feb25 MP samples. Figure 4.8 summarizes how the reads were assigned across different taxonomic levels. In most samples, assignments remained strong at the order and family level. The decrease observed at the species level is typical for amplicon-based or metagenomic workflows, since closely related taxa can share highly similar marker regions that limit resolution.

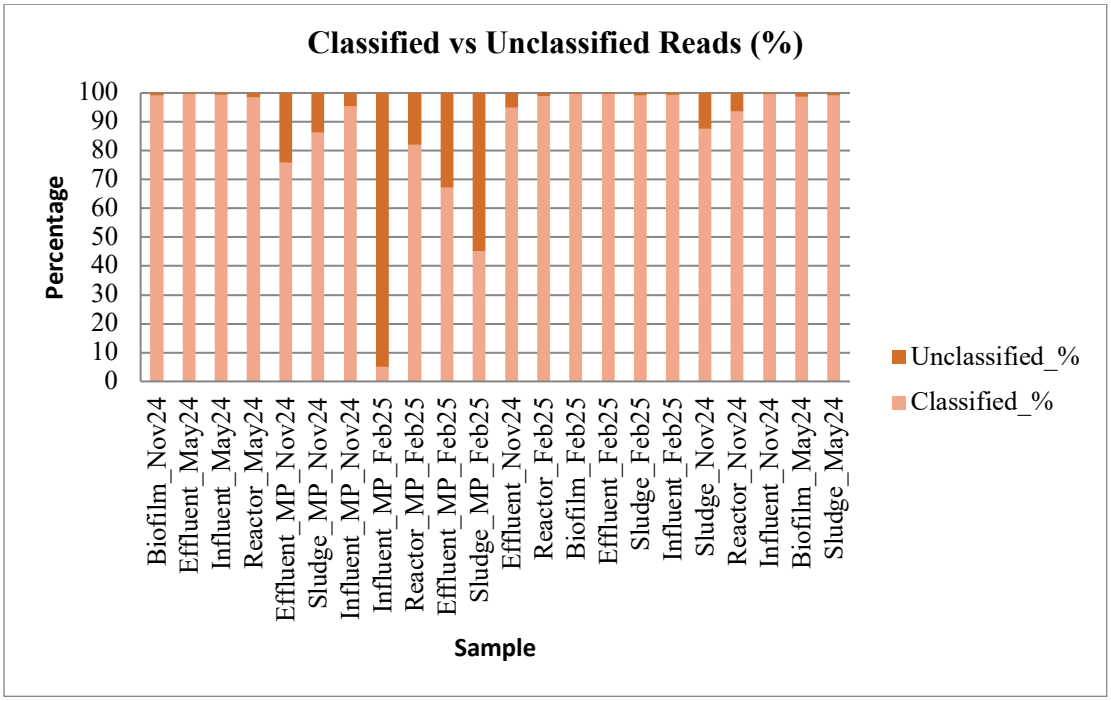


Figure 4.7. Percentage of classified and unclassified sequencing reads across all samples.

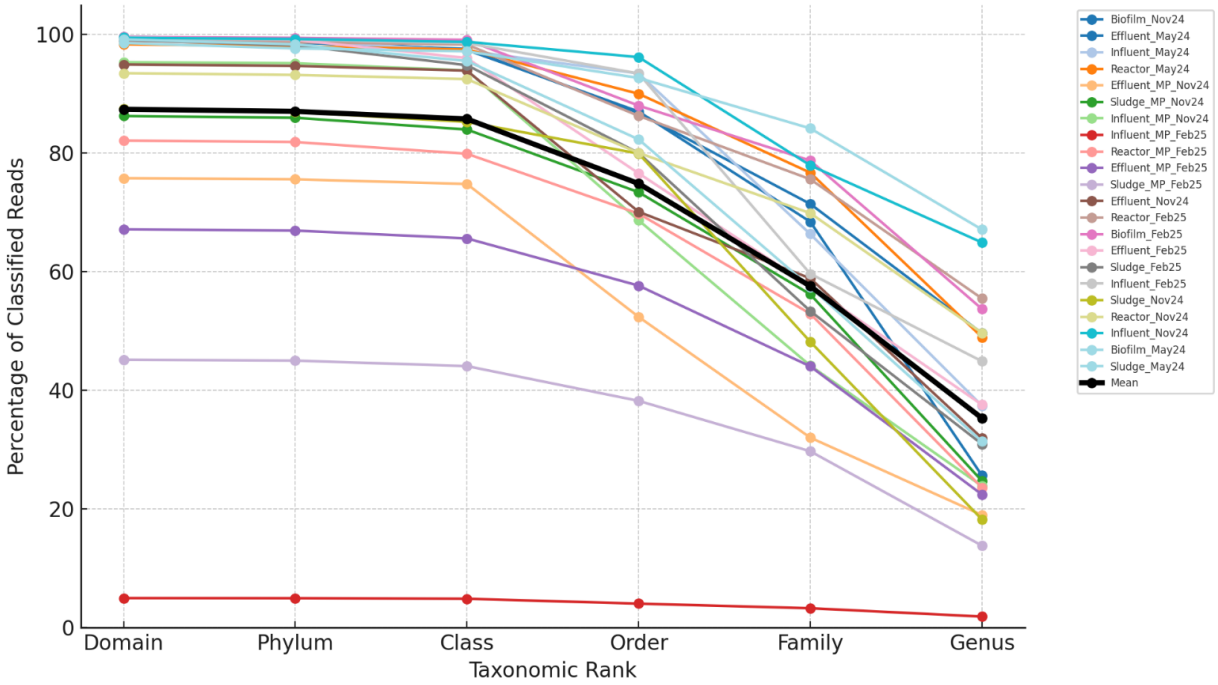


Figure 4.8. Percentage of classified reads retained across taxonomic ranks (domain to genus).

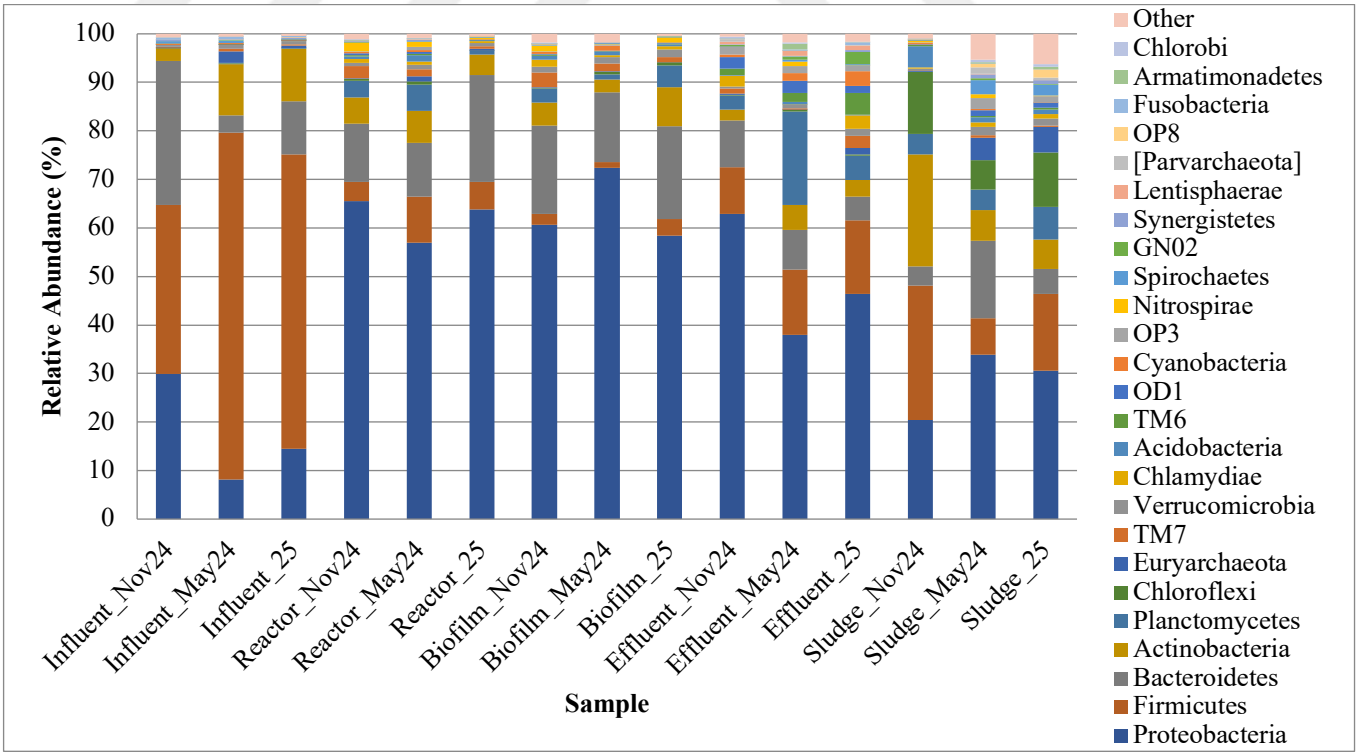


Figure 4.9. Relative abundance of dominant bacterial phyla across all bulk samples collected in May24, Nov24, and Feb25.

As shown in Figure 4.9, the microbial community structure in the bulk environmental samples shows considerable variation across different sampling dates (Nov24, May24, and Feb25), indicating temporal shifts in the influent characteristics rather than seasonal precipitation effects, since the plant is a closed system, receiving no storm water but only sewage. *Proteobacteria, Firmicutes*, and *Bacteroidetes* consistently dominate across almost all sample types and dates, although their relative abundances vary considerably.

Across the three sampling periods, the influent exhibited significant shifts in its microbial community structure, reflecting the variable nature of the incoming wastewater rather than any seasonal precipitation effect, as the plant operates as a closed system, as mentioned previously. *Proteobacteria, Firmicutes*, and *Bacteroidetes* consistently dominated the influent, although their relative abundances fluctuated markedly between May24, Nov24, and Feb25, with *Actinobacteria* remaining a recurrent sub-dominant group. For example, *Firmicutes* shifted from 71.5% (May24) to 34.8% (Nov24) and rose again to 60.7% (Feb25), while *Proteobacteria* varied from (8.2% in May24), then increased substantially (29.9% in Nov24), only to decrease again (14.4% in Feb25). *Bacteroidetes* followed a similar pattern *to Proteobacteria*, starting low (3.6% in May24), peaking (29.6% in Nov24), and then decreasing (10.9% in Feb25). Such fluctuations are well aligned with previous findings showing that WWTP influents typically receive highly heterogeneous microbial loads from households and institutional sources, resulting in substantial temporal variability in dominant phyla (Witsø et al., 2024). Similar dominance patterns of *Proteobacteria*-rich communities have been widely reported in bulk wastewater matrices (Galafassi et al., 2021). Overall, the marked fluctuations observed in this study likely reflect upstream changes in wastewater composition rather than environmental seasonality, consistent with literature emphasizing influent characteristics as the primary driver of microbial variation entering WWTPs (Witsø et al., 2024).

The reactor tank exhibited a far more stable microbial community compared to the influent, indicating the system's strong buffering capacity. *Proteobacteria* were consistently the dominant phylum in all three sampling periods (May24: 57.0%, Nov24: 65.6%, Feb25: 63.8%), which aligns with previous studies showing that wastewater treatment systems typically host *Proteobacteria*-rich communities, accompanied by *Bacteroidetes, Firmicutes*, and *Actinobacteria* as secondary groups (Kong et al., 2024). *Firmicutes* showed relatively modest fluctuations (9.4% in May24, 3.9% in Nov24, and 5.7% in Feb25), whereas *Bacteroidetes* increased notably in Feb25 (11.2% in May24, 11.9% in Nov24, and 22.0% in

Feb25). *Planctomycetes* displayed a consistent decline (5.5% in May24, 3.4% in Nov24, 0.7% in Feb25), suggesting a gradual ecological shift within the reactor environment.

The biofilm communities displayed a distinct and consistently *Proteobacteria*-dominated profile across all sampling periods (May24: 72.4%, Nov24: 60.6%, Feb25: 58.5%). This pattern is characteristic of attached-growth systems, where aerobic and facultative *Proteobacteria* are strongly favored due to their metabolic versatility and ability to form stable surface-associated structures (Greenwich et al., 2024). *Bacteroidetes* represented the second most abundant phylum, consistently ranging from 14-19%, while *Actinobacteria* showed moderate fluctuations (4.7% in May24, 2.6% in Nov24, ans 8.0% in Feb25). *Planctomycetes* varied more prominently, decreasing between May24 and Nov24 (2.8% to 1.1%) before increasing again in Feb25 (4.5%), suggesting shifts in niche availability during biofilm maturation.

The effluent community shows significant variability, reflecting the cumulative effects of treatment processes and likely influenced by biomass detachment from the reactor and biofilm. *Proteobacteria,* while dominant in each sampling period, exhibited substantial variation (37.9% in May24, rising to 62.9% in Nov24, then decreasing to 46.4% in Feb25). This mirrors the trend in the reactor, suggesting effective removal. *Chloroflexi* showed a significant increase in Feb25 (11.19%) compared to earlier samples (0.36% May24 ,0.11% Nov24). The significant changes in dominant phyla, particularly the large swings in *Proteobacteria* and the rise of *Planctomycetes* and *Chloroflexi* in the final sample (Feb25), suggest that the treatment process's ability to consistently remove or manage certain microbial groups in the outflow might be fluctuating.

The sludge community exhibited a composition distinct from the overlying water and biofilm samples, reflecting the anaerobic and fermentative conditions characteristic of settled biomass. *Firmicutes* constituted a major component of the community across all sampling periods, showing an increase from May24 to Nov24 and remaining relatively high in Feb25. *Proteobacteria* and *Bacteroidetes* also contributed substantially, while *Chloroflexi* displayed pronounced enrichment, increased from 6% (May24) to 12.9% (Nov24), then slightly decreased to 11.2% (Feb25). The significant changes in *Firmicutes, Proteobacteria*, and *Bacteroidetes*, along with the notable presence of *Euryarchaeota* and *Chloroflexi* (which are often associated with anaerobic digestion and organic matter degradation (Kirkegaard et al., 2017)), indicate that the sludge community was responding to the variable inputs from the influent and potentially the treatment processes.

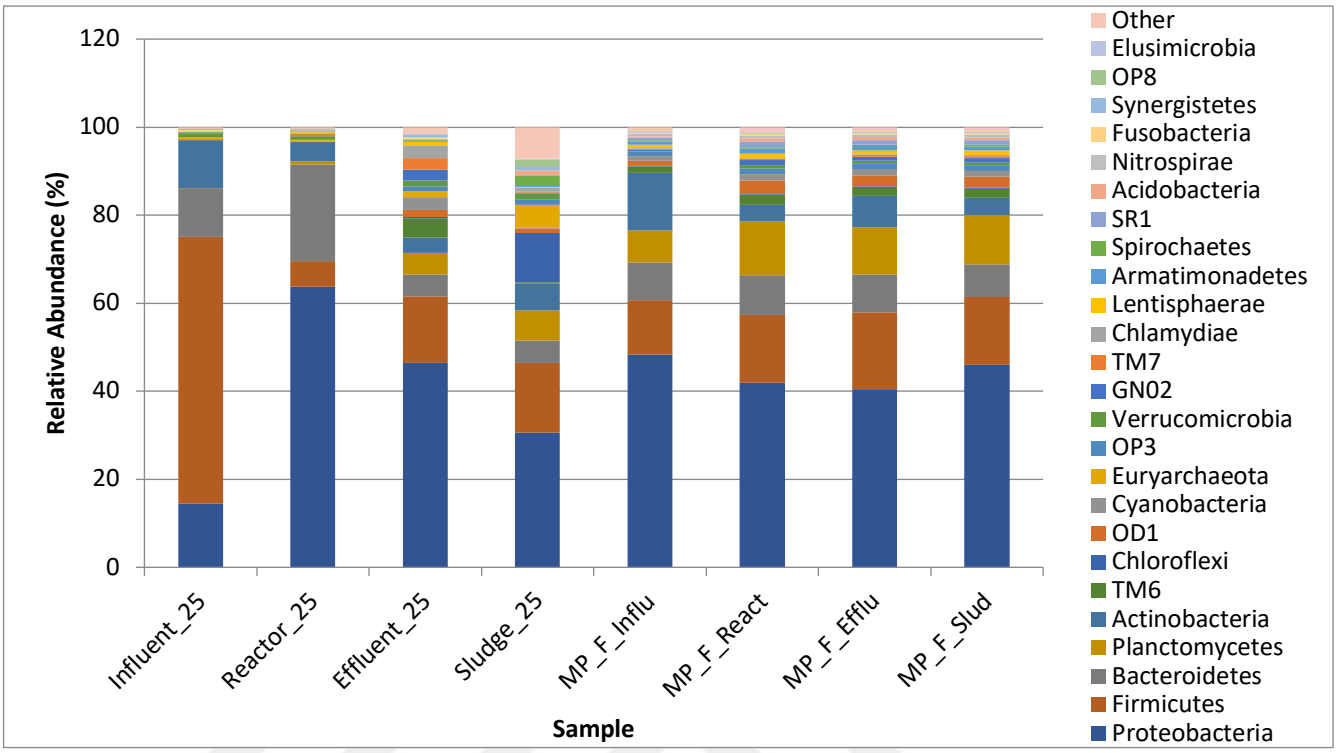


Figure 4.10. Phylum-level microbial community composition in bulk samples and microplastic-associated fractions (Feb25).

Figure 4.10 illustrates the phylum-level microbial compositions across all bulk sample types and their corresponding microplastic-associated fractions. *Proteobacteria* and *Firmicutes* constitute two of the major phyla in the system; however, their dominance shifts substantially depending on the sample matrix. In the influent, *Firmicutes* is the most dominant phylum, representing 60.7% of the total community, while *Proteobacteria* accounts for about 14%. However, in the reactor tank, there is a significant change where *Proteobacteria* becomes overwhelmingly dominant at approximately 64%, while *Firmicutes* drops to about 6%, indicating strong selective pressure or growth advantage for *Proteobacteria* in the reactor conditions. *Bacteroidetes* consistently represents a substantial portion of the community, ranging from about 11% in influent to 22% in reactor, 4.9% in effluent, and 5.2% in sludge. *Chloroflexi* shows a remarkably low presence in influent, reactor, and effluent (0.1-0.3%), but it is relative abundance significantly increases in sludge (11.2%), reflecting the anaerobic niche typical of settled biomass (Kirkegaard et al., 2017). *Actinobacteria* shows variability, being around 11% in influent, dropping to 4.2% and 3.4% in reactor and effluent, respectively.

In the bulk influent sample (Influent_25), the community is dominated by *Firmicute*s, which constitute 60.7% of the total composition. *Proteobacteria* represent a smaller portion (14.44%), while *Bacteroidetes* (10.97%) and *Actinobacteria* (10.91%) also appear at notable levels. Other groups such as *Planctomycetes, TM6* and *Chloroflexi* are present only in trace amounts. However, this pattern changes on the microplastic-associated fraction (In MP_F_Influent). Here, *Proteobacteria* become the dominant phylum, increasing to 48.34%, while *Firmicutes* decline sharply to 12.4%. *Actinobacteria* increase slightly, and several low-abundance phyla in the bulk water particularly *Planctomycetes, TM6* and *Chloroflexi*, show clear enrichment on the microplastic particles. *Bacteroidetes* decrease modestly but remain part of the community. Overall, the influent shows important changes in community structure on microplastics, marked by the enrichment of *Proteobacteria* and several typically rare phyla, and a strong reduction in *Firmicutes* compared to the surrounding water. This pattern is consistent with earlier reports showing that microplastic surfaces favor early colonizing *Proteobacteria* and enrich rare phyla such as *Planctomycetes* and *TM6*, which are often underrepresented in bulk wastewater communities (Bydalek et al., 2023).

In the bulk reactor sample (Reactor_25), *Proteobacteria* form the dominant phylum at 63.8%, reflecting the selective conditions of the RBC tank, where oxygen availability and continuous shear tend to favor aerobic and facultative surface-associated taxa (Bydalek et al., 2023). *Bacteroidetes* also constitute a substantial portion of the community at 22%, whereas *Firmicutes* and *Actinobacteria* appear at much lower levels (5.7% and 4.20%, respectively). Only trace amounts of *Planctomycetes, TM6,* and *Chloroflexi* are present in the surrounding water. The microplastic-associated fraction shows a different pattern. *Proteobacteria* remain the most abundant group but decrease to 41.92%, while *Firmicutes* become more prominent on the particles, rising to 15.36%. *Bacteroidetes* decrease substantially to 9.13%, and several typically low-abundance phyla, most notably *Planctomycetes,* which reach 12.17%, show clear enrichment on microplastics. *TM6* and *Chloroflexi* also increase compared to the bulk community. Overall, the reactor displays a shift where *Proteobacteria* remain dominant in the surrounding water, but *Firmicutes*, *Planctomycetes, TM6,* and *Chloroflexi* are more strongly represented on the microplastic surfaces.

In the bulk effluent sample (Effluent_25), *Proteobacteria* form the largest portion of the community at 46.4%, followed by *Firmicutes* at 15.2%. *Planctomycetes* and *TM6* appear at 5.0% and 4.4%, respectively, which is relatively high compared to some of the other bulk samples. *Bacteroidetes* (4.9%) and *Actinobacteria* (3.4%) are present at moderate levels, while *Chloroflexi* remain very low. On the

microplastic-associated fraction, *Proteobacteria* continue to dominate but drop slightly to 40.5%, whereas *Firmicutes* increase to 17.5%. *Planctomycetes* show a clear enrichment on microplastics, rising to 10.7%, and both *Bacteroidetes* and *Actinobacteria* also increase in relative abundance. In contrast, *TM6* declines sharply on the particles compared to its level in the bulk water. *Chloroflexi* remain at low levels in both fractions. Overall, the effluent shows a pattern where *Proteobacteria* stay dominant across fractions, but *Firmicutes, Planctomycetes, Bacteroidetes* and *Actinobacteria* are more represented on microplastic surfaces, while *TM6* becomes less abundant.

In the bulk sludge sample (Sludge_25), *Proteobacteria* constitute the largest portion of the community at 30.6%, followed by *Firmicutes* at 15.8%. *Chloroflexi* are particularly notable in this matrix, reaching 11.2%, which is higher than in any of the other bulk sample types. *Planctomycetes* (6.8%), *Actinobacteria* (6%), and *Bacteroidetes* (5.2%) also contribute to the community, while *TM6* remains at very low levels. The microplastic-associated fraction shows a different pattern: *Proteobacteria* become even more dominant, rising to 45.9%, and *Firmicutes* remain nearly unchanged (15.5%). *Planctomycetes* show clear enrichment on microplastics, increasing to 11.2%, and *Bacteroidetes* and *TM6* also increase in relative abundance. In contrast, *Actinobacteria* decrease on the particles, and *Chloroflexi* show a striking decline, dropping from over 11% in the bulk to only 0.3% on microplastics. Overall, the sludge samples indicate that *Proteobacteria, Firmicutes,* and *Planctomycetes* tend to accumulate on microplastic surfaces, whereas *Chloroflexi* are strongly depleted.

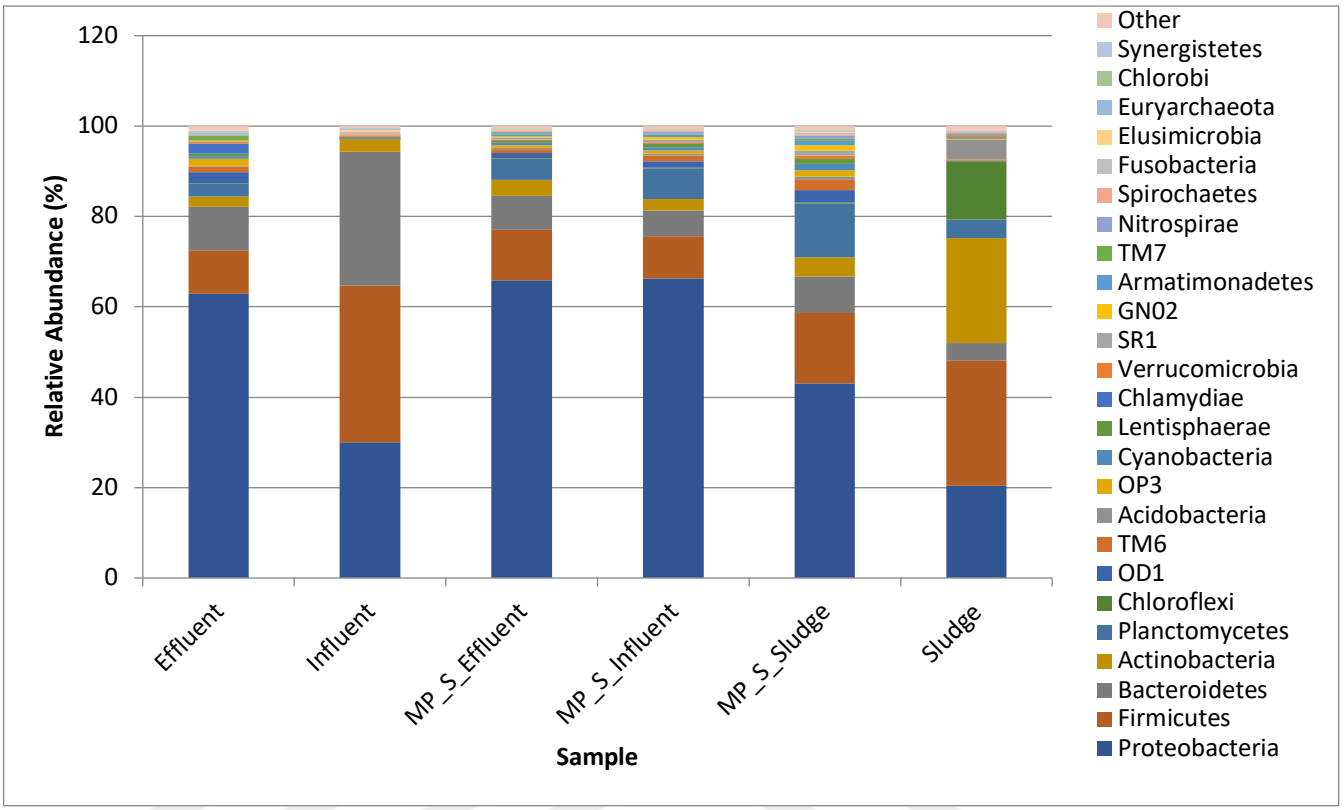


Figure 4.11. Relative abundance of microbial phyla in bulk and microplastic-associated fractions (Nov24)

Figure 4.11 illustrates the phylum-level composition of the microbial communities across the influent, effluent, and sludge samples belonging to the Nov24 sampling date. In the influent, the microbial community was primarily dominated by *Firmicutes* (34.8%), *Proteobacteria* (30%), and *Bacteroidetes* (29.6%), which together accounted for the majority of the microbial structure. This distribution reflects typical upstream wastewater inputs, where fecal and gut-associated groups such as *Firmicutes* and *Bacteroidetes* are prevalent, while *Proteobacteria* remain consistently abundant due to their metabolic versatility (Tuyen et al., 2025; Zhong et al., 2023). Following treatment, the bulk effluent showed a distinct shift with *Proteobacteria* becoming overwhelmingly dominant (62.9%), while *Firmicutes* (9.6%) and *Bacteroidetes* (9.7%) significantly decreased in relative abundance compared to the influent. The bulk sludge displayed a different profile, characterized by a more even distribution among *Proteobacteria* (20.4%), *Firmicutes* (27.7%), and notably, a high abundance of *Actinobacteria* (23.1%). *Chloroflexi* also showed a significant presence in sludge (12.9%).

The microbial community associated with microplastics in the influent (MP_S_Influent) was already largely dominated by *Proteobacteria* (66.3%), even more so than the bulk influent, indicating strong initial colonization preference or rapid growth of *Proteobacteria* on MP surface upon entry into system. Conversely, *Firmicutes* were the most abundant phylum in the bulk influent at 34.78%, showing a significant decrease to only 9.34% on the microplastic-association fraction (MP_S_Influent), indicating a strong depletion or competitive disadvantage on microplastic surfaces in this initial stage. While almost negligible in the bulk influent (0.03%), *Planctomycetes* showed a significant enrichment on the microplastic-association fraction, reaching 6.73%. Overall, microplastics in the influent act as a selective surface, strongly favoring the colonization by *Proteobacteria* and *Planctomycetes*, while significantly reducing the relative abundance of *Firmicutes* and *Bacteroidetes* compared to the surrounding water column. The strong dominance of Proteobacteria on microplastics in the influent is consistent with earlier observations that this phylum rapidly attaches to plastic surfaces and often becomes the initial driver of plastisphere formation (Bhardwaj et al., 2025; M. Xu et al., 2024).

Both bulk effluent (62.9%) and the microbial community associated with microplastics in the effluent (MP_S_Effluent) (65.9%) showed high relative abundances of *Proteobacteria*. The slight increase in the MP_S_Effluent suggests their continued dominance and perhaps a slightly enhanced presence on microplastics even after treatment. The relative abundance of *Firmicutes* remained low in both bulk effluent (9.6%) and MP_S_Effluent (11.1%), though there was a marginal increase on the MP surface. *Bacteroidetes* slightly decreased on MP_S_Effluent (7.5%) compared to the bulk effluent (9.7%). Similar to the influent, *Planctomycetes* showed a noticeable enrichment in MP_S_Effluent (4.7%) compared to the bulk effluent (2.9%). There was a modest enrichment of *Actinobacteria* on MP_S_Effluent (3.6%) compared to bulk effluent (2.2%). While *Proteobacteria* dominate both environments, microplastics in the effluent continue to show a subtle enrichment of *Planctomycetes* and *Actinobacteria*, suggesting these phyla are maintained or even favored on the plastic surface post-treatment. The consistently high abundance of Proteobacteria on microplastics in the effluent aligns with reports that this phylum remains a dominant and resilient colonizer of plastic surfaces even after biological treatment (Martínez-Campos et al., 2021).

The sludge environment also shows distinct patterns when comparing the bulk community with the MP-associated fraction. In the bulk sludge, *Proteobacteria* accounted for 20.42% of the community. However, on the MP_S_Sludge, their relative abundance increased markedly to 43.1%, indicating a continued preference or greater survival rate for *Proteobacteria* on microplastic surfaces within the

sludge matrix. *Firmicutes* were quite abundant in the bulk sludge at 27.7%. On the MP_S_Sludge, their abundance significantly decreased to 15.6%, suggesting they are less dominant on microplastics in this environment compared to the bulk sludge. While relatively low in bulk sludge (3.9%), *Bacteroidetes* showed a notable increase in the MP_S_Sludge, reaching 8.1%. This indicates a positive association with microplastics in the sludge environment. *Actinobacteria* were a major component of the bulk sludge, representing 23.1%. However, on the MP_S_Sludge, *Actinobacteria* drastically declined to 4.2%, suggesting a strong selective disadvantage or displacement on microplastic surfaces within the sludge. Similar to the influent and effluent, *Planctomycetes* showed significant enrichment on MP_S_Sludge (11.9%) compared to the bulk sludge (4.2%). Overall, in the sludge phase, microplastics continue to attract *Proteobacteria, Bacteroidetes*, and *Planctomycetes*, while significantly repelling or inhibiting the growth of *Actinobacteria* and *Firmicutes* compared to the surrounding sludge matrix. This suggests that even within a highly complex environment like sludge, microplastics act as a distinct ecological niche. These patterns partially agree with previous observations showing that *Proteobacteria* and *Bacteroidetes* commonly associate with or persist on microplastics in WWTPs, while other phyla display more variable responses depending on local sludge conditions (Kong et al., 2024).

5. CONCLUSIONS

This study provides a comprehensive assessment of microplastic abundance, characteristics, removal behavior, and microbial colonization in a full-scale WWTP employing an RBC attached-growth system. Seasonal sampling revealed that microplastic inflow loads varied across the year, most likely reflecting changes in campus population and daily activities, whereas effluent concentrations remained consistently low. Across all sampling events, fibers were the dominant microplastic type, and medium-sized particles (250–1000 μm) constituted the largest fraction. The RBC system demonstrated high removal efficiencies (92–96%) regardless of seasonal fluctuations, highlighting its robustness in microplastic retention. Sludge acted as the primary sink for microplastics, while biofilm formed on RBC disks also contributed to retention, albeit to a lesser extent.

Polymer characterization showed that PET and nylon were the dominant polymers across samples, with smaller amounts of PP, PVC, PPSU, and PVBP. The prevalence of PET, nylon, PP, and PVC based fibers indicates that most particles originated from textile sources such as washing machine effluents, while the occasional detection of PPA is likely associated with abrasion of internal equipment within the closed RBC system. Microbial community analyses revealed that MP-associated assemblages differed from bulk water and sludge communities, indicating that microplastics support distinct microbial colonization patterns. Dominant phyla included *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Chloroflexi*, aligning with taxa commonly reported in wastewater environments but exhibiting shifts in relative abundance depending on sample type and season.

Overall, the findings highlight the capacity of attached-growth systems such as RBCs to effectively capture microplastics while simultaneously generating microplastic-associated microbiomes that differ from the surrounding environment. The combined evaluation of microplastic fate and microbial colonization provides new insights into the intersection of particle removal performance and microbial ecology in WWTPs. These results may support future optimization of attached-growth systems and contribute to broader strategies aimed at mitigating microplastic release into aquatic environments from WWTPs.

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