Impact of pollution sources of microplastics and associated microbial populations in surface water

by

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APPROVAL

I hereby approve the final submission of the following dissertation.

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DECLARATION

I hereby declare that this thesis entitled "**Impact of pollution sources on microplastics and associated microbial populations in surface water**"

1. Is my original work and has not been submitted for a degree at any other university. Its only prior publication was in the form of a journal article.
2. I further declare that a detailed reference list has been provided on all cited literature and resources.

04/12/2024

Signature of student Date

09/12/2024

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07/12/2024

Signature of co-supervisor Date

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ABSTRACT

Microplastics (MPs) are ubiquitous environmental pollutants of global concern, presenting a major threat to aquatic ecosystems. The study examined the effects of potential pollution sources of MPs and associated microbial communities in riverine environments, including wastewater treatment plants (WWTPs), agricultural areas (AA), urban areas (UA), and industrial discharge (IA). The study sites were selected along the uMsunduzi River in KwaZulu-Natal, and the sampling was conducted in two seasons (summer and winter). Morphological and chemical characterization of MPs was performed using microscopy, ATR-FTIR, and Pyro-GC/MS analysis. Shotgun metagenomics was used to analyze the microbial community. The potential health risks associated with selected pathogens in the biofilm were also assessed using Quantitative Microbial Risk Assessment (QMRA).

Microplastics were detected in abundance from all four sites with concentrations in the IA being the highest (69 particles/L), followed by the WWTP (51 particles/L), the UA (49 particles/L), and the AA (39 particles/L). Additionally, sediment samples showed higher MP particles compared to the surface water. The most common types of MP detected were fibers, followed by pellets and fragments for both surface water and sediment samples. Furthermore, the key polymers detected via chemical characterization were polyethylene (PE), Polyethylene terephthalate (PET), polypropylene (PP), polystyrene (PS), and Polyvinyl Alcohol (PVA) across all sites with varying dominance. The PS, PET, and PE were predominant at the UA, while the WWTP and IA exhibited a variety of polymers, including PE, PP, PET, and PS. The AA site showed the presence of PE, PP, PS, PET, and PVA.

Metagenomic data demonstrated a significant microbial diversity ($p = 0.0012$) and composition (PERMANOVA $F = 16.386$; $R2 = 0.15$, $p < 0.001$) in different sites (UA, WWTP, AA, and IA), and habitat (surface water and plastisphere). The plastisphere harbored a distinct microbial

community compared to surface water. At the phylum level, *Bacteroidetes* were significantly higher in surface water, whereas α- and *β-Proteobacteria* dominated on the plastic surface ($p < 0.05$). In regard to the different sites, WWTP had the most different taxa (5), followed by UA (3), with AA and IA each having only 1 unique taxon. The distance decay model showed that microbial communities in the plastisphere and surrounding environments are significantly positively associated with the sources of pollution (UA: $R^2 = 0.83$, $p = 0.015$; WWTP: $R^2 = 0.88$, $p = 0.0072$; AA: $R^2 = 0.85$, $p = 0.0075$; IA: $R^2 = 0.95$, $p = 0.0011$).

The study also revealed the presence of various antimicrobial resistant genes (ARGs) in both surrounding surface water and plastisphere, with MP surfaces showing higher ARGs than surrounding surface water. For instance, the plastisphere harbored 19 ARGs compared to 9 in surface water. The WWTP showed diverse ARGs, including the widely reported ARGs conferring resistance to tetracycline, fluoroquinolone, and aminoglycoside. The study also identified 17 pathogenic microbial species across different sites, with *Acinetobacter baumannii* being the most dominant. Furthermore, common pathogens such as *Escherichia coli, Pseudomonas aeruginosa, Acinetobacter baumannii,* and *Klebsiella pneumoniae* were detected across all sites, seasons, and habitats. The microbial risk assessment based on two dominant pathogens (*Pseudomonas aeruginosa* and *Salmonella enterica*) revealed that the risk of infection varied across different pollution sources and seasons. Notably, the highest infection risk associated with selected pathogens was found in IA and WWTP-impacted sites which is in accordance with the total number of MPs detected indicating and increase in MPs will have a significant impact on the associated health risks. Results of this study indicate that different pollution sources significantly influence MP abundance and types, as well as the structure of microbial communities, which may ultimately pose a threat to human health.

PREFACE

The outputs of the Master's qualification include:

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Publications

- MUNEER AHMAD MALLA, INDHUR RIONA, **MALAMBULE NL**. **NOMALIHLE**, MOSAGALE KELEBOGILE, MOODLEY TYRONE, BUX FAIZAL,

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- **MALAMBULE L. NOMALIHLE**, ISAAC D. AMOAH, MALLA A MUNEER, MOODLEY TYRONE, NNADOZIE C, KUMARI SHEENA, 2024. Riverine microplastics: Unravelling the pollution puzzle from source to sediment.
- MALLA A. MUNEER, **MALAMBULE L. NOMALIHLE**, ISAAC D. AMOAH, JONATHAN FEATHERSTON, ARSHAD ISMAILA, FAIZAL BUX, SHEENA KUMARI, 2024.Comprehensive profiling and risk assessment of antibiotic resistomes in surface water and plastisphere by integrated shotgun metagenomics. Journal of Hazardous Materials (HAZMAT-D-24-16910). (**First and Second authors contribute equally**)

Contributions

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LIST OF ABBRIVIATIONS

PP:	Polypropylene
PET:	Polyethylene terephthalate
PVC:	Polyvinyl chloride
ABS:	Acrylonitrile butadiene styrene
PU:	Polyurethanes
PE:	Polyethylene
PLA:	Polylactic acid
PC:	Polycarbonate
PS:	Polystyrene
PMMA:	Polymethyl Methacrylate
MP:	Microplastics
NP:	Nanoplastics
HGT:	Horizontal Gene Transfer
ARGs:	Antimicrobial-resistant genes
MGEs:	Mobile genetic elements
AMR:	Antimicrobial resistance
DO:	Dissolve Oxygen
TN:	Total nitrogen
TP:	Total phosphorus
WWTP:	Wastewater treatment plants
UA:	Urban area
AA:	Agricultural area
IA:	Industrial area

PS:	Plastisphere
HDPE:	High-density polyethylene
LDPE:	Low-density polyethylene
PUR:	Polyurethane
PHA:	Polyhydroxyalkanoates
GPS:	Global positioning system
TDS:	Total dissolved solids
NH_3:	Ammonium nitrogen
NO_2:	Nitrite nitrogen
NO_3:	Nitrate nitrogen
PO_4^{3-}:	Phosphate
COD:	Chemical oxygen demand
dH_2O:	Distilled water
H_2O_2:	Hydrogen peroxide
NaCl:	Sodium Chloride
ATR-FTIR:	Attenuated total reflectance Fourier transform infrared spectroscopy.
Py- GC/MS:	Pyrolysis-gas chromatography-mass spectrometry
AB:	Absorption band
PCCP:	Personal care and cleaning products
EPS:	Extracellular polymeric substance
SEM:	Scanning electron microscopy
CLSM:	Confocal laser scanning microscopy

CHAPTER I

1.0 INTRODUCTION

1.1 BACKGROUND

Plastic polymers are widely used in all aspects of our daily lives owing to their low cost, durability, lightweight, and good ductility. Plastic production exceeded 400.3 million metric tons in 2022, resulting in an increase of about 1.6 percent from the previous year according to Annual Production of Plastics Worldwide from 1950 to 2022 (Statista, 2022). Polypropylene (PP), polyethylene terephthalate (PET), polyvinyl chloride (PVC), acrylonitrile butadiene styrene (ABS), polyurethanes (PU), polyethylene (PE), polylactic acid (PLA), polycarbonate (PC), and polystyrene (PS) are frequently used plastic materials and are commonly found in aquatic ecosystems (Mammo *et al*., 2020, Dong *et al*., 2021, Kaur *et al*., 2021). They have been detected in a variety of environments such as seawater, lakes, rivers, wetlands, glaciers, icebergs, streams, ponds, marshes, ice sheets, and groundwater. In the environment, they degrade into smaller particles such as micro and nanoplastics (MP and NP) (Nava and Leoni, 2021, Alprol *et al.*, 2021, Gola *et al*., 2021, Syranidou and Kalogerakis, 2022) which are also called as secondary plastics. The microplastics that are made intentionally in smaller sizes for industrial purposes are called primary microplastics (Naik *et al*., 2019, Gola *et al*., 2021). This include microbeads used in personal care products, acrylic textile fibers released from washing clothes, and industrial blast cleaning etc (Naik *et al.*, 2019). Secondary MPs originate from the gradual weathering of larger plastics in the environment through mechanical factors (wind and wave action), temperature changes, biological processes (microbial degradation), and chemical degradation (UV-B radiation in the environment) (Naik *et al*., 2019, Alprol *et al*., 2021).

Microplastics are also known to serve as potential substrates for microbial attachment and biofilm formation. These biofilms are composed of one or more biological communities, such as bacteria, fungi, algae, and protozoans (Yang *et al.*, 2020c, Kaur *et al*., 2021). Studies have shown that microbes forming biofilms on MP surfaces are significantly different from those found in the surrounding environment and in natural particles (Miao *et al.*, 2019, He *et al*., 2022b). These biofilms, collectively known as plastisphere, create a novel ecological niche on microplastic surfaces, fostering unique microbial assemblages (Zettler *et al*., 2013, Kirstein *et al*., 2018). Bacterial taxa, such as *Pirellulaceae, Phycisphaerales, Cyclobacteriaceae, Flavobacteriaceae, Rhodobacteraceae, Planctomycetaceae,* and *Phyllobacteriaceae*, as well as several alpha- beta-Proteobacteria, *Bacteroides, Actinobacteria, Patescibacteria, Firmicutes*, and *Cyanobacteria*, are commonly associated with MPs (Kaur *et al*., 2021). The microbial communities associated with MPs also contain pathogenic organisms such as *Vibrio, Acinetobacter, Xanthomonas*, and as well as bacteria associated with MP degradation, such as *Pseudomonas, Klebsiella*, and *Sphingomonas* (Pinto *et al*., 2019, Yang *et al*., 2020c, Kaur *et al.*, 2021). A higher abundance of *Vibrio spp*. has been reported on MP surfaces compared to surrounding water (Dong *et al.*, 2021). Similarly, other pathogens, such as *Arcobacter spp*., a human pathogen, plant pathogens such as *Agrobacterium spp*.; nosocomial pathogens such as *Chryseobacterium spp*.; and fish pathogens such as *Flavobacterium spp*. are reported in low-density polyethylene biofilms (Yang *et al.*, 2020c, Kaur *et al*., 2021).

Additionally, microplastic particles have been found to influence microbial community evolution and increase gene exchange between phylogenetically diverse microbes via horizontal gene transfer (HGT) (Arias-Andres *et al.*, 2019, Pinto *et al*., 2019, Kaur *et al.*, 2021, Stenger *et al.*, 2021). HGT facilitates the transfer of antimicrobial-resistant genes (ARGs) between the biofilm-forming microbes and ambient bacteria via various mobile genetic

elements. This increases the likelihood that microplastic-associated biofilms will acquire ARGs from distant environments and, thereby promote the transfer of pathogenicity (Kaur *et al*., 2021). This has increased research interest due to their ecological and environmental impacts on aquatic environments, ecosystems, and human health (Stenger *et al*., 2021). The accumulation of ARGs and drug-resistant bacteria in biofilms is found to carry sulphonamide-resistant genes (sul1 and sul2) that persist in the aquatic environment (Syranidou and Kalogerakis, 2022). These antimicrobial resistance (AMR) genes and mobile genetic elements (MGEs) are abundant in long-time exposed MPs containing biofilms of *Pseudomonas, Syntrophomonas*, and *Desulfotomaculum* in wastewater landfill leachate, with intra-bacterial MGE transfer capabilities and increasing environmental risks (Kaur *et al.*, 2021).

The biofilm communities in aquatic environments are found to be influenced by various factors, including the availability of nutrients (organic and inorganic carbon, nitrate, and phosphorus) for microbial growth, pollutants (toxic metals, antibiotics, and persistent organic pollutants), physicochemical parameters (dissolved oxygen (DO), light, pH, temperature, salinity, and ionic strength), and aquatic biota (plants and animals) (Yang *et al*., 2020c, Rajcoomar, 2023). For instance, biofilm development is observed to be more uniform and thinner when the carbon source is unstable and there is no substantial cell interaction (Harrison *et al*., 2020). Similarly, salinity appears to have a significant impact on the bacterial diversity of biofilms attached to MP surfaces in aquatic environments (Yang *et al*., 2020c, He *et al*., 2022b). Nutrients (total nitrogen (TN) and total phosphorus (TP)) are positively correlated with biofilm growth rate, whereas salinity has a negative relationship (He *et al*., 2022b). Seasonal changes were also found to have an impact on the biofilm community. For example, it has been reported that the biofilms growing on the surface of MPs have more biomass and a faster growth rate in the summer (Wu *et al*., 2019b). Furthermore, the abundance of

autotrophs in biofilms is also reported influenced by DO content, where lower levels of DO result in higher chlorophyll formation (Wu *et al.*, 2019b, Yang *et al.*, 2020c, He *et al.*, 2022b).

1.2 Research Problem

The uMsunduzi River faces various pollution challenges, including different pollution sources (wastewater treatment plant discharge, industrial waste discharge, agricultural and urban runoff) which contribute to the presence of MPs in surface water. These MPs, depending on their size and density as well as the flow rate of the river, either get transported or accumulate in the river sediments. MPs serve as surfaces for the attachment of microbial pathogens and antibiotic-resistant genes, facilitating their transport to various locations. However, there is a lack of knowledge regarding the role that different sources of pollution play in influencing the type and distribution of MPs, the water quality, and the type of pathogens and antimicrobial resistance genes attaching to their surfaces in the uMsunduzi River. Understanding the impact of these pollution sources is crucial for developing effective strategies to mitigate the environmental and health risks associated with microplastic pollution in this river system.

1.3 AIM AND OBJECTIVES

1.3.1 Aim

The aim of this study was to examine the impact of different pollution sources on surface water quality, the type of microplastics, the distribution of microplastic particles and associated biofilm communities in surface water.

1.3.2 Objectives

- To assess the impact of different pollution sources on the water quality based on the physicochemical analysis using a yellow spring instrument and nutrient analysis kit.
- To isolate and characterize microplastic particles from surface water samples using light microscopy.

- To analyze the diversity of microbial communities associated with MP surfaces using shotgun metagenomic sequencing analysis.
- To assess health risks imposed by potential pathogens associated with MPs in surface water using quantitative microbial risk assessment

CHAPTER II

2.0 LITERATURE REVIEW

2.1 Global Plastic Production

Plastic products have proven to be a great asset to modern life because of their practical qualities and economic cost (Asamoah *et al*., 2019, Europe, 2020). Annually, global plastic production has been steadily growing since the 1950s, with more than 400 million metric tonnes of produced annually as of recent years (Shaibur *et al*., 2024). This figure is expected to reach 500 million metric tonnes by 2025, and projections suggest it could soar to 34 billion metric tonnes by 2050 according to Europe (2020). This exponential growth underscores the increasing reliance on plastics in various industries and everyday applications, highlighting the urgent need for sustainable management and innovative solutions to address the ensuing environmental challenges.

Owing to its lack of management and limited biodegradability, plastic waste has become a major environmental issue (Geyer *et al.*, 2017, Krüger *et al.*, 2020). Geyer *et al*., (2017) discovered that approximately 79% of the total amount of plastic waste was released into the environment, 12% was burnt, and only 9% was recycled. The ubiquity of plastic pollution in society is due to the widespread use of plastic items across nearly all industries (Chen *et al*., 2021). In South Africa, the plastic industry has shown signs of growth and recovery in recent years. According to the South African Plastic Pact, only 43.2% of the total waste that reached the market was recycled in 2020, while 81.2% of the total packaging plastics that reached the market were recycled in 2021. The packaging sector remains the largest consumer of plastic polymers in South Africa, accounting for 50% of the total consumption, with rigid packaging

at 29% and flexible packaging at 21%. The building and construction sector follows at 14% (Malematja *et al.*, 2023).

2.2 Plastic size and type

The amount of plastics present in the aquatic ecosystem depends on various factors including the residence time, size, and types of plastics, as well as the sources of plastic pollution and their vertical distribution within the water column (Uddin *et al.*, 2020). Different types of plastics such as polyethylene terephthalate (PET), low-density polyethylene (LDPE), high-density polyethylene (HDPE), polyurethane (PUR), polyvinyl chloride (PVC), polycarbonate (PC), polymethyl methacrylate (PMMA), Acrylonitrile Butadiene Styrene (ABS), polyamide (Nylon), polystyrene (PS), and polypropylene (PP) (Table 2.1) contribute to pollution due to their chemical diversity and density variations (Naik *et al.*, 2019)**.** The densities of these plastics affect their buoyancy and distribution, influencing whether they remain on the water surface, are suspended in the water column, or sink to the bottom. For example, less dense plastics like LDPE and HDPE are more likely to float, while denser plastics such as PET and PVC tend to sink. This vertical distribution, coupled with the types and sizes of plastics, determines the extent and impact of plastic pollution in aquatic environments (Uddin *et al.*, 2020). Understanding these dynamics is crucial for addressing the pervasive issue of plastic pollution and developing effective mitigation strategies (Uddin *et al.*, 2020).

Table 2 1: Polymer type, density, and their uses

Polymer type	Density	Properties	Uses	Reference
LDPE	0.91–0.93 g/cm³	Flexible, tough, transparent, good impact resistance	Plastic bags, film wraps, squeeze bottles, toys	(Lwanga *et al.*, 2018, Uddin *et al.*, 2020)

HDPE	0.94–0.97 g/cm³	Strong, durable, resistant to impact and chemicals, opaque	Milk jugs, detergent bottles, piping, plastic lumber	(Bringer *et al.*, 2020, Uddin *et al.*, 2020)
PP	0.90–0.91 g/cm³	Hard, resistant to chemicals and heat, fatigue-resistant, lightweight	Packaging, automotive parts, textiles, medical	(Alsabri *et al.*, 2022, Uddin *et al.*, 2020)
PVC	1.16–1.58 g/cm³	Rigid or flexible, durable, resistant to chemicals and weathering, can be made softer with plasticizers	Pipes, window frames, flooring, cables, medical devices	(Uddin *et al.*, 2020, Kudzin *et al.*, 2023)
PS	1.04–1.10 g/cm³	Lightweight, rigid, or foamed, can be clear, good insulation properties	Disposable cups, plates, food containers, insulation	(Kik *et al.*, 2020, Uddin *et al.*, 2020)
PET	1.38–1.41 g/cm³	Strong, lightweight, resistant to moisture, clear	Beverage bottles, food containers, synthetic fibers	(Uddin *et al.*, 2020, Dhaka *et al.*, 2022)

PC	1.20–1.22 g/cm³	High impact resistance, transparent, resistant to heat, but not scratch-resistant	Eyewear lenses, medical devices, electronic casings	(Uddin *et al.*, 2020, Qin *et al.*, 2021)
PMMA	1.16–1.20 g/cm³	Transparent, strong, resistant to UV light and weathering, easily moldable	Windows, skylights, illuminated signs, aquariums	(Uddin *et al.*, 2020, Dong *et al.*, 2022)
Nylon	1.13–1.15 g/cm³	Strong, flexible, resistant to wear and chemicals, can be processed into fibers	Textiles, automotive parts, machinery, packaging	(Uddin *et al.*, 2020, Mejías *et al.*, 2023)
ABS		Tough, impact-resistant, easily moldable, opaque	LEGO bricks, electronic housings, automotive components	(Uddin *et al.*, 2020, Hu *et al.*, 2022)

Aforementioned plastics in the environment can be divided into four categories, based on size (Figure 2.1): Mesoplastics are defined as plastic particles with a size of 5mm to 2.5 cm. Macroplastics are those that are >2.5 cm, microplastics (MPs) range from 1µm to 5 mm (Alprol *et al.*, 2021). Nanoplastics (NPs) are minuscule particles that are nearly invisible to the eye,

with the diameter of their particles ranging from 1 to 10 nanometers (0.001 to 0.1 micrometers) (Boyle and Örmeci, 2020, Alprol *et al*., 2021). They can be found in face washes, soap products, and many other consumer products. Generally, MPs are identifiable by their morphological characteristics such as shape, size, and color (Cole *et al*., 2013, Alprol *et al*., 2021). Factors such as shape (e.g. spheres, fragments, fibers, and film) (Figure 2.2, Table 2.2) and size (primary and secondary particles) play an important role in distinguishing between microplastics (Alprol *et al*., 2021).

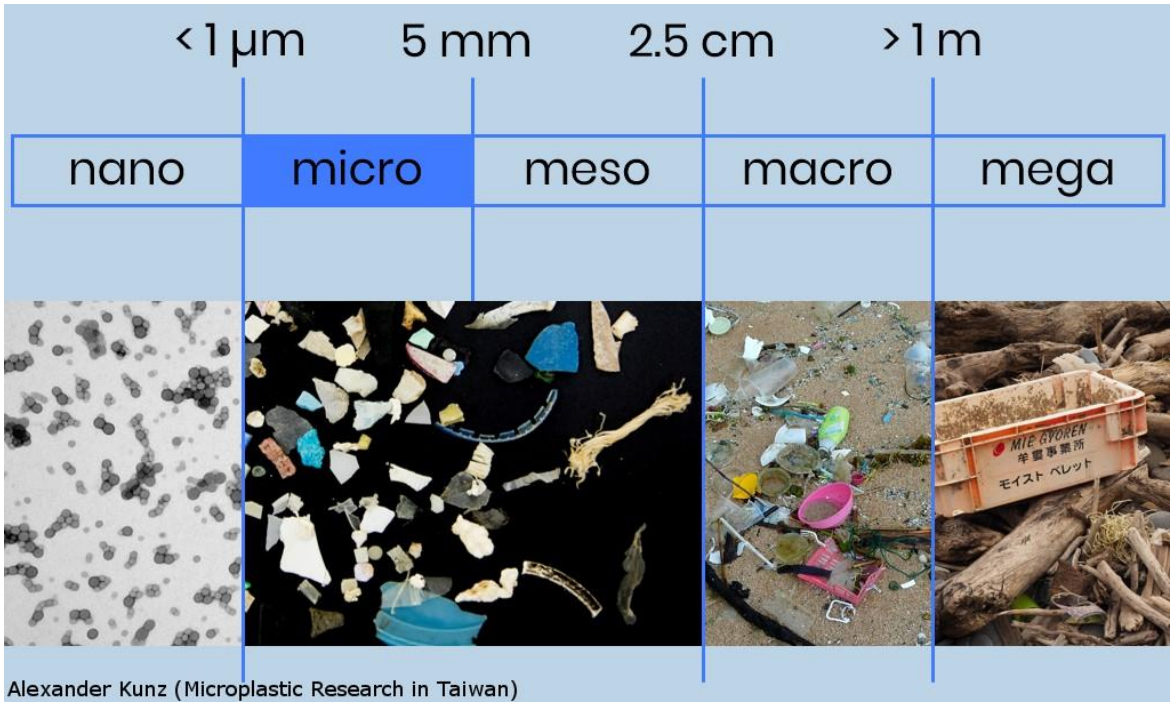


Figure 2. 1: Characterization of microplastic in different plastic sizes (Kunz, 2018).

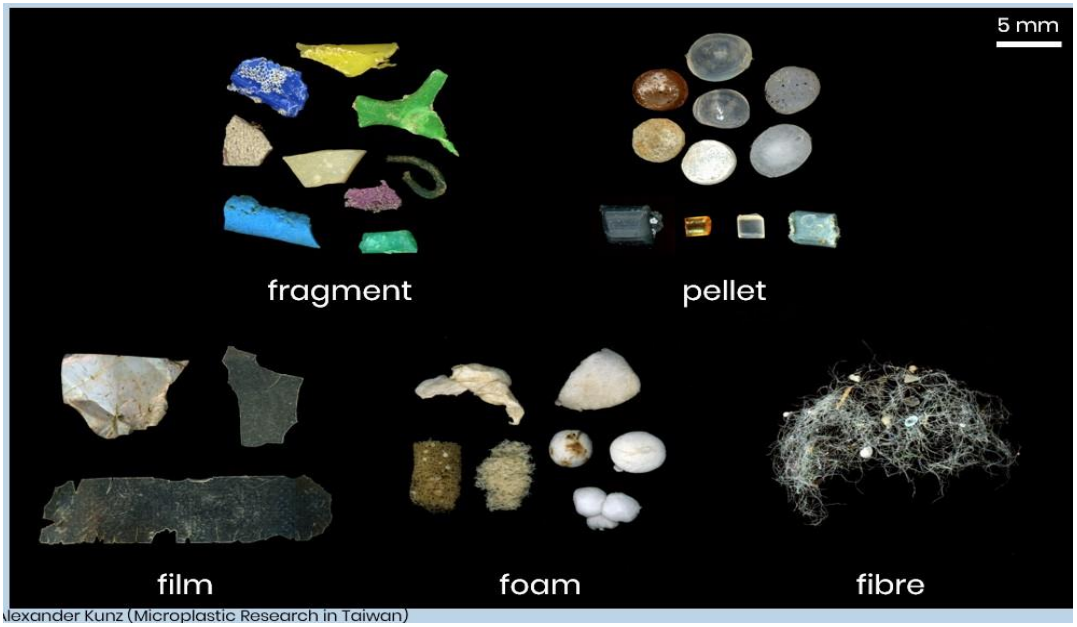


Figure 2. 2: Characterization of microplastic in terms of shape (Kunz, 2018).

Table 2. 2: MPs with descriptive shapes

Fragment	Particles are derived from larger plastic pieces. They are available in a wide range of shapes and colors. By far the most common shape is fragments.	(Kunz *et al.*, 2016, Andrady, 2017)
Pellet	Particles derived from industrial resin pellets. They can be cylindrical or spherical depending on their state of weather. They are most commonly white translucent, but they can also be a wide range of other colors.	(Kunz *et al.*, 2016, Andrady, 2017)
Film	Plastics that are thin, flexible, and sheet-like. They are typically made of plastic bags, plastic foil, or other	(Kunz *et al.*, 2016,

	packing materials. It can be difficult to tell the difference between a thin, sheet-like fragment and a plastic film.	Andrady, 2017)
Foam	Any plastic that has a foamed structure. Styrofoam or other expanded or foamed plastics such as PS, PE, or PVC could be used.	(Kunz *et al.*, 2016, Andrady, 2017)
Fiber	Any fibrous plastic will do. Clothing washing generates a large portion of synthetic fibers. Correctly assessing synthetic fibers in a sample can be difficult because they can come from secondary contamination in the air or clothing of those who process the samples.	(Kunz *et al.*, 2016, Andrady, 2017)

2.3 Classification based on origin

2.3.1 Primary MPs

Primary MPs are defined as plastics that are produced intentionally for industrial and domestic applications. This includes plastic particles found in cosmetics, such as shower/bath gels, scrubs, peels, eye shadows, deodorants, blush powders, makeup foundations, mascara, shaving cream, baby products, bubble bath lotions, hair dye, nail varnish, insect repellents, and sunscreen (Auta *et al*., 2017), as well as in air blasting media, synthetic garments, abrasives used in cleaning supplies, and drilling fluids (Cole *et al*., 2011). These consumer products are classified as "open use", because they are meant to be washed off and end up in drains. An increasing number of reports have also been made regarding the use of MPs as drug-delivery systems in medicine (Cole *et al*., 2011, Auta *et al.*, 2017). Although their inclusion in this category has been criticized (Auta *et al*., 2017), virgin plastic production pellets (typically 2-5

mm in diameter) can also be considered as primary MPs under the broader size definitions of a microplastic (Auta *et al*., 2017, Wang *et al*., 2021d).

2.3.2 Secondary MPs

Secondary MPs are small plastic fragments (micro or nano-sized particles) originating from larger plastic garbage degradation in the environment (Auta *et al*., 2017, Chamas *et al*., 2020). The structural integrity of plastic debris can be compromised over time owing to a combination of physical, biological, and chemical processes, leading to fragmentation (Chamas *et al*., 2020). Exposure to sunlight over long periods of time can cause photodegradation of plastics; UV radiation promotes the oxidation of the polymer matrix, which leads to link cleavage. As a result of this degradation, additives designed to improve durability and corrosion resistance may seep out of the polymer and ends up in the environment (Cole *et al.*, 2011, Auta *et al*., 2017, Chamas *et al.*, 2020).

2.3.3 Source of microplastics in the aquatic environment

Microplastics in aquatic ecosystems primarily originate from land-based plastic debris (Auta *et al.*, 2017). Plastics are generally created and used on land, yet a substantial amount of plastic debris and MPs escape, often traveling through rivers and ending up in marine habitats (Figure 3) (Wang *et al.*, 2021d). Annually, it is estimated that eight million tonnes of plastic pollution enter the oceans, with 80% originating from land-based sources (Wang *et al*., 2020b, Wang *et al.*, 2021d). Edo *et al*. (2020) estimated that 300 million pieces of MPs were discharged into rivers on a daily basis. Furthermore, runoff, erosion, and wind transfer contribute to transport of MPs from terrestrial to aquatic habitats (Edo *et al*., 2020, Kaur *et al.*, 2021).

Industrial waste is a significant contributor to microplastic pollution (Chen *et al*., 2021, Kaur *et al*., 2021). Manufacturing processes, including plastic production and textile manufacturing, release MPs directly into wastewater systems, with studies showing that textile washing

processes and tire wear particles contribute substantially to aquatic microplastic pollution (Lambert and Wagner, 2018, De Falco *et al.*, 2018). Additionally, improper disposal and inadequate recycling of industrial plastic waste lead to fragmentation into MPs (Prata *et al.*, 2020). Similarly, agricultural sectors also contribute to MP particles, and runoff from agricultural fields add to the microplastic load in surface waters (Napper, 2018, Fan *et al.*, 2022).

Sewage and WWTPs are the major sources of MPs released into aquatic systems, especially those in urban areas (Edo *et al*., 2020). The burgeoning tourist industry is also instrumental in contributing to the influx of plastics into aquatic habitats (Chen *et al.*, 2021, Kaur *et al.*, 2021). Tourists often leave behind plastic items such as plastic bags, mineral water bottles, and food wrappings that are discarded into water bodies, such as rivers, lakes, seas, coasts, and oceans (Wang *et al.*, 2018, Chen *et al.*, 2021). In addition, shipping activities spread MPs into water by discarding plastic waste from vessels (Chen *et al.*, 2021). Moreover, the natural degradation of plastic products, caused by light, temperature fluctuation, and the erosive action of freshwater or seawater further adds to the stockpiling of MPs in the water (Wang *et al.*, 2021d, Kaur *et al.*, 2021).

Atmospheric deposition of MPs is another input that has recently attracted increasing attention (Murphy *et al.*, 2016, Wang *et al.*, 2021d, Chen *et al.*, 2021). Although urban environments have been associated with greater levels of airborne MPs, the proximity of coastal cities to water sources may result in higher MP levels in rivers and oceans (Chen *et al.*, 2021). Long-range atmospheric movement play a significant role in the concentration of MPs in the Arctic region (Bergmann *et al*., 2019). Overall, the prevalence of these particles, especially in far-off places such as the arctic, is indicative of the scope of MP pollution (Chen *et al.*, 2021, Wang *et al*., 2021d).

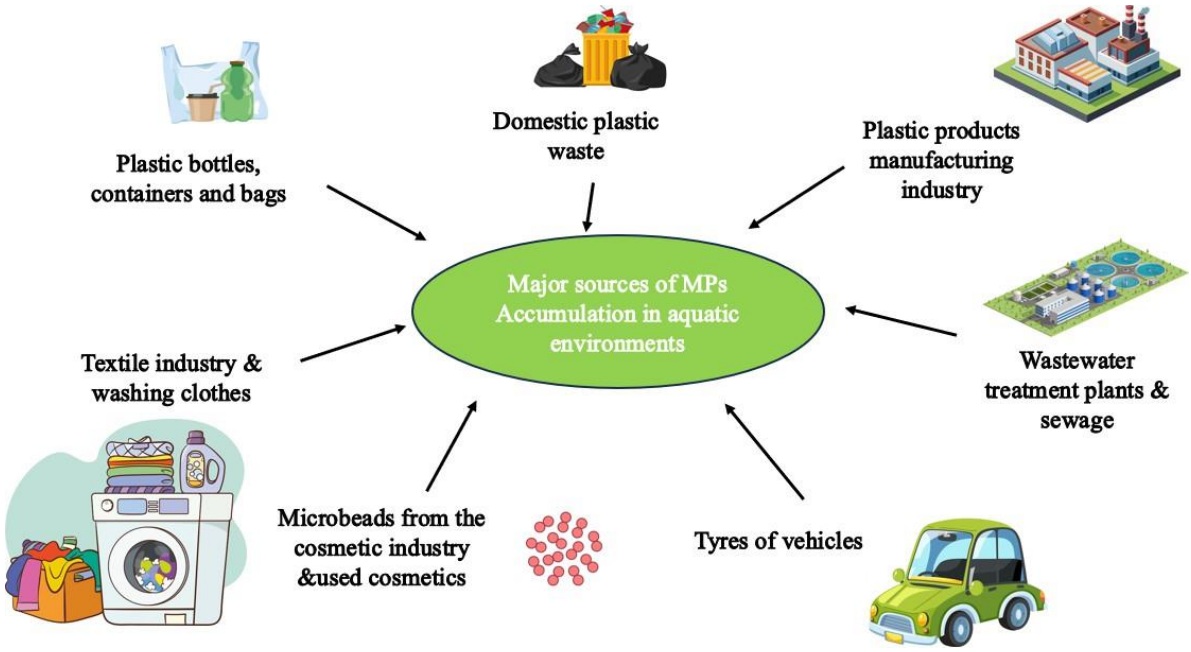


Figure 2. 3: Major sources of MPs into aquatic environments (Katare *et al*., 2021).

2.4 Transport of microplastics in the aquatic environment

Numerous natural ecosystems, such as coastal areas (Ballent *et al*., 2012, Zhang *et al.*, 2017, Kumar *et al.*, 2021), mountain basins (Allen *et al.*, 2019), and river basins (Nizzetto *et al.*, 2016) have demonstrated the transfer and placement of MPs in sediment and surface water samples (Wang *et al.*, 2021a). A study has been conducted to determine the fate, transportation, and removal of MPs in distinct aquatic habitats (Boyle and Örmeci, 2020, Kumar *et al.*, 2021). Rivers continue to emerge as important sources of MPs to the ocean both through transport and retention, but quantitative evidence for this is currently limited (Kumar *et al.*, 2021). They can act as temporary sinks that delay the release of MPs to the oceans, but when rain events occur, rivers can become swift transport as the flow rate increases (Cole *et al.*, 2011). The transportation of MPs in the aquatic environment is subject to many natural processes, such as biofouling, aggregation, flocculation, and degradation, which can significantly alter their environmental fate (Ballent *et al*., 2012, Auta *et al*., 2017, Bilal and Iqbal, 2020). Additionally,

changes in particle size, composition, and shape profoundly affect the transport, deposition, and accumulation of MPs within aquatic systems, largely because of differences in the respective densities, mass, and surface heterogeneity (Andrady, 2017, Wang *et al*., 2021a). For example, larger MPs may be retained in riverbed sediments (Nizzetto *et al*., 2016) whereas smaller particles may remain afloat in surface water. Furthermore, the physical interactions between MPs and the environment are largely dependent on their shape, size, and density (Cole *et al.*, 2011, Anderson *et al.*, 2016, Horton and Dixon, 2018), highlighting the complexity associated with the fate and transport of MPs in the river environment (Horton and Dixon, 2018, Boyle and Örmeci, 2020, Kumar *et al*., 2021).

The interaction between MPs and the natural environment has an intense effect on the monitoring of transport and deposition in riverine ecosystems (Horton and Dixon, 2018). Investigations have been conducted on microplastic pollution in various rivers around the world. For example, studies on the Ganga River along the Dehradun, India have documented a MP concentration ranging between 2 800 to 4 200 items/L for surface water while for sediment ranging between 7 200 to 16 400 items/kg highlighting severe pollution (Nayal and Suthar, 2022). Research in the Seine River in Paris has reported a median MP concentration of 15.5 items/L with an interquartile of 4.9 items/L, with significant variations based on location and season (Treilles *et al.*, 2022). Natural conditions such as wind, surface runoff, and rainfall significantly influence the transportation of MPs in rivers. Studies demonstrated that wind can disperse microplastics over distances of up to 50 km with a concentration in affected coastal areas varying significantly with wind speed and direction(Allen *et al.*, 2019, Bruge *et al.*, 2020) Surface runoff and rainfall events have been shown to increase micr5oplastic concentrations in river by 35% during heavy rainfall, underscoring the role of weather patterns in microplastic transport (Lang *et al.*, 2020). Air currents also contribute to the dispersion of MPs, with concentrations in the atmosphere ranging from 2 to 10 particles/ m2/day in urban

areas, depending on local airflow and pollution sources (Cai *et al*., 2017). Additionally, flooding events can lead to significant spikes in number of MPs in river systems, with increases of up to 40% observed during and after major flood events (Roebroek *et al*., 2021). Overall, these statistical findings illustrate the complex dynamics of MP pollution and transport in aquatic environments (Blair *et al*., 2017). This occurs owing to downward movement and is exacerbated by the advection flow of plastic particles in the longitudinal direction (Blair *et al*., 2017). This was evidenced in Brazil, where domestic sources along the river basin combined with increased river flow during high rain events led to a high level of solid waste, including plastics, on beaches (Blair *et al*., 2017). The Danube River was found to be an important route of plastic production from Germany and Austria to the Black Sea, and it is widely assumed that fluctuations in floating densities were due to the proximity of production plants (Blair *et al*., 2017). Additionally, evidence of higher microplastic layers in two Chicago urban rivers occurred only during wet periods and rain events, mostly of the unregulated primary MNP that were discharged into oceans (Blair *et al.*, 2017).

2.6 Biofilm formation on microplastics

Microplastics exist as suspended particles that absorb high abundances of organic nutrients from the surrounding environments which result in the attraction of microbial colonizers utilizing the nutrients (Oberbeckmann *et al*., 2015, Wang *et al*., 2021c). Due to the hydrophobic surface of plastics, microbes are able to create a biofilm or "plastisphere" that serves as an environmental niche (Wang *et al*., 2021c). This allows for significant interactions between microorganisms and their surroundings, which contain a variety of inorganic and organic materials (Wang *et al.*, 2021b, Syranidou and Kalogerakis, 2022). Furthermore, buoyancy and stability provided by MPs offer a secure foothold for microorganisms, resulting in their extended survival over extended periods of time. These microbial communities comprise

heterotrophic and autotrophic species as well as predators, symbionts, eukaryotes, and bacteria (Harrison *et al*., 2018, He *et al.*, 2022b). The population of microorganisms found in MPs tends to be higher than that in the water itself. The resistance of MPs to decay results in the transportation and migration of parasites and pathogens across multiple ecosystems and watercourses (Harrison *et al*., 2018).

2. 6.1 Colonization of Microplastics surfaces by biofilm.

MPs can collect nutrients and organic materials from their surroundings because they are suspended particles. This supports the basic survival and reproduction of microbes that colonize MP surfaces (Wang *et al.*, 2021c). In addition, the presence of sufficient organic resources it provides microorganisms with a stable environment to resist environmental pressure and accelerate dispersion (Wang *et al*., 2021b, Wang *et al*., 2021c). Microplastics are ubiquitous particles, they are found everywhere including aquatic, terrestrial, and atmospheric habitats (Lin *et al*., 2020, Kaur *et al*., 2021). Seawater is the most studied environment for MP-colonizing microflora. It has been reported that bacteria quickly colonize MPs in this environment within minutes to hours (Quero and Luna, 2017). Furthermore, Dang *et al*. (2008) discovered that microorganisms attached to MP surfaces in a sequential order rather than randomly or accidentally throughout the colonization process. For instance, Lee *et al*. (2008) found that *Proteobacteria* dominated the microbial community during the early phases of colonization in marine biofilm ecosystems. After 12 h, the secondary biofilm microflora consists of a *Proteobacteria* community. The variety of plastisphere communities is typically influenced by elements including substrate type, seasonal and geographical functions (Gong *et al.*, 2019, He *et al.*, 2022b).

Cai *et al*. (2019) compared the adherence of microbes to four different types of particles and discovered that surface roughness was the primary factor influencing the strength of bacterial adherence to PE and PVC. According to Gong *et al*. (2019), the assembly of bacterial

communities on LDPE is influenced by the hydrophobicity and roughness of the particles (Wang *et al.*, 2021c). Additionally, microbial communities are affected by changes in the external environment in addition to variations in substrates (Cai *et al.*, 2019, Wang *et al.*, 2021b). According to Amaral-Zettler *et al*. (2015), bacterial populations vary with latitude and are relatively distinct between ocean basins. Variations in the microflora linked to plastics are also caused by environmental conditions such as salinity, pressure, oxygen, and the movement of suspended particles (Amaral-Zettler *et al.*, 2015, Harrison *et al*., 2018). Most significantly, harmful bacteria can quickly colonize MP surfaces (Kirstein *et al*., 2016, Huang *et al*., 2019, Khalid *et al*., 2021).

2. 6. 2 The uniqueness of microplastic biofilms

Over time, biofilm communities develop on the surfaces of the polymer materials. Research has shown that microbial communities found on the surfaces of MPs differ considerably from those found in the surrounding surface water (Zettler *et al.*, 2013, Wu *et al.*, 2020). Wu *et al.* (2020) revealed that plastics held an increased presence of specific families like *Halobacteriaceae* and *Pseudoalteromonadaceae* and lessened geographical discrepancies in samples. Moreover, plastic-degrading groups such as *Actinobacteria, Bacteroidetes*, and *Proteobacteria* are more common in MP biofilms than expected (Miao *et al*., 2019, Wang *et al*., 2021c). Controlling factors, such as the distinctive physicochemical characteristics of MP debris, are responsible for the discrepancies in relative abundance. According to Miao *et a*l. (2019), species inhabiting marine plastic materials present lower alpha diversity (in terms of richness, evenness, and diversity) than biofilms thriving on natural substrates. McCormick *et al*. (2016) similarly noted a considerably decreased alpha diversity in biofilms adhering to MPs when compared to those upon setons and other water column substrates. Conversely, Dussud *et al*. (2018) concluded that MP biofilms featured greater levels of evenness and taxonomic heterogeneity in comparison to free-living and organic particle-anchored materials. It appears

that these apparent contradictions can be linked to the various available environmental resources, as well as the existing spatial heterogeneity (McCormick *et al*., 2016, Dussud *et al*., 2018, Wang *et al*., 2021c).

Recent investigations utilizing gene-targeted metagenomics and shotgun metagenomics have produced detailed information regarding the composition of microbial communities found on MPs (Amaral-Zettler *et al.*, 2015, De Tender *et al.*, 2015, Bryant *et al.*, 2016, Pinnell and Turner, 2019, Pinnell and Turner, 2020). According to research by Bryant *et al.* (2016), De Tender *et al*. (2015), and Zettler *et al*. (2013), microbial communities associated with beach pellets can differ from the microbes found in the surrounding environment or even those existing in natural particles in the same environment (De Tender *et al.*, 2015, Ogonowski *et al.*, 2018, Miao *et al*., 2019, Wu *et al*., 2019b). De Tender *et al.* (2015) revealed that the main outcome for these variations is often the comparatively higher abundance of *Actinobacteria* on beach pellets compared to *Proteobacteria* which are more prevalent in sediments and seawater (Sathicq *et al*., 2021).

2.7 Factors influencing microbial attachment

2.7.1 Microplastics' properties

Plastics come in various forms because they are frequently used. The following are some of the findings from research comparing biofilms on various MPs: (i) Biofilms grown on PP and PS microplastics showed tighter aggregation (Frère *et al.*, 2018); (ii) compared with HDPE and, higher bacterial abundance was observed on LDPE (Xu *et al.*, 2019b). The surface energy, surface roughness, hydrophobicity, and other features of MPs largely influence the diversity of the microorganisms (He *et al.*, 2022b). The impact of the different forms of MPs is particularly pronounced in the early phases of biofilm growth (Wang *et al.*, 2021b, He *et al.*, 2022b).

Varying charges on the surfaces of different types of MPs may have different effects on bacterial adherence (Wang *et al.*, 2021b, Kaur *et al.*, 2022). For instance, research has shown that the negatively charged nature of PE and PS microplastics makes them less conducive to bacterial adhesion (Wang *et al.*, 2021c). The cell membrane of gram-negative microbes may be harmed by some MPs' positive surface charges. In addition, as the surface roughness of MPs increases, so does their surface area and preference for microbes, creating a more inviting environment for them to adhere (Wang *et al*., 2021b, He *et al.*, 2022b). The MP surfaces concentrate the nutrients scattered throughout the environment and offer sufficient nutrients for the development of biofilms (Hossain *et al.*, 2019, Zhao *et al.*, 2021b). Furthermore, the adherence of microorganisms is affected by variance in nutrient richness on the substrate surface. *Salmonella* species and *Escherichia coli* adhere more tenaciously to nutrient-deficient substrates than *L. monocytogenes* and *Staphylococcus sciuri* due to nutrient-rich substrates, according to earlier research (He *et al*., 2022b).

2.7.2 The characteristics of affiliating microbes

The composition of microbial cells is crucial in the early stages of microbial colonization (Wang *et al.*, 2021b). According to previous studies, many bacterial organelles play significant roles in the adhesion process (He *et al.*, 2022b). For instance, flagella are essential for the early stage of reversible adhesion, which explains why strains lacking flagella are less capable of adhering to surfaces and forming biofilms (Wang *et al.*, 2021b, He *et al.*, 2022b). Many microorganisms are part of the biofilm communities, and due to the variety of species and their interaction, they form complicated dynamics of synergy and competition. Some biofilms can grow more productive and persistent than the original colonized colony; synergistic interactions between microbial communities are key to this (He *et al.*, 2022b). A foundation for collaboration between various microorganisms, biofilms act as substrates for the interchange of numerous metabolites, signalling chemicals, and genetic material (Harrison *et*

al., 2018). Pre-colonizing microbes may prevent the colonization of other organisms by preventing intercellular communication or by decomposing polysaccharides and proteins (Wang *et al.*, 2021c, He *et al.*, 2022b).

Several of the bacteria that predominate in MPs have quorum sensing systems, which are communication mechanisms between bacteria (Harrison *et al.*, 2018). Bacteria regulate the development and growth of biofilms by creating and responding to signalling molecules (Harrison *et al.*, 2018, Xu *et al.*, 2019a). As a result, the variety of later colonizing microbes in the biofilms will be influenced by the early colonizing microbes. For instance, *Acinetobacter calcium*, may serve as a link in the biofilm creation process and automatically collect some bacteria to produce copolymers. Hence, one of the variables determining the production of biofilm is microbial characteristics (Wang *et al.*, 2021b, He *et al.*, 2022b).

2.7.3 Environmental factors

Biofilm communities vary in various aquatic settings, with key elements influencing development, including flow state, nutrient availability, seasonal shifts, and pH. First, fluctuations in flow rate can have a considerable effect on biofilm formation. Rapid flows have been found to reduce the colonization rate and end-state coverage, although the latter is thinner and denser (Kaur *et al.*, 2022). Carbon, nitrogen, and phosphorus form the essential nutritional building blocks for successful development, with a greater and more regular growth rate observed in carbon-poor conditions, with minimal strain adhesion. Moreover, the biofilm development rate has been confirmed to correlate positively with nutrients (total nitrogen (TN) and total phosphate (TP)) while salinity has a converse effect (Figure 5) (Li *et al.*, 2020a, Kaur *et al.*, 2022).

Seasonal variations in temperature, light, and dissolved oxygen can impact the growth and development of biofilms on the surface of MPs. Generally, summer has a higher temperature, longer illumination time, and lower dissolved oxygen content, driving up the metabolic rate

and enzyme activity of cells (Zeraik and Nitschke, 2012, Chen *et al.*, 2019a, He *et al.*, 2022b). As a result, throughout the summer, biofilms developing on MP surfaces have higher biomass and a faster development rate (He *et al.*, 2022b). Similarly, reduced dissolved oxygen levels are often accompanied by increased chlorophyll concentrations, which results in the abundance of autotrophs in biofilms (Wang *et al.*, 2021b, He *et al.*, 2022b). The adhesion of microorganisms to the substrate is determined by both van der Waals forces and Coulomb forces (Kaur *et al.*, 2022). The former is typically an attractive force whereas the latter involves either attraction or repulsion caused by electrical layer overlap between the cells and the surface. However, the pH of a given environment has a profound effect on dominant interactions (Yang et al., 2020b). As a result, adhesion dynamics were altered. Different bacteria have varying preferences with respect to pH, meaning that species composition and bacterial abundance fluctuate depending on whether the environment is acidic, neutral, or alkaline in nature (He *et al.*, 2022b). In turn, this impacts the intricate characteristics of biofilm growth, including shape, coverage, and growth rate. In conclusion, a multitude of factors will ultimately determine the development of a biofilm (Wang *et al.*, 2021b, He *et al.*, 2022b).

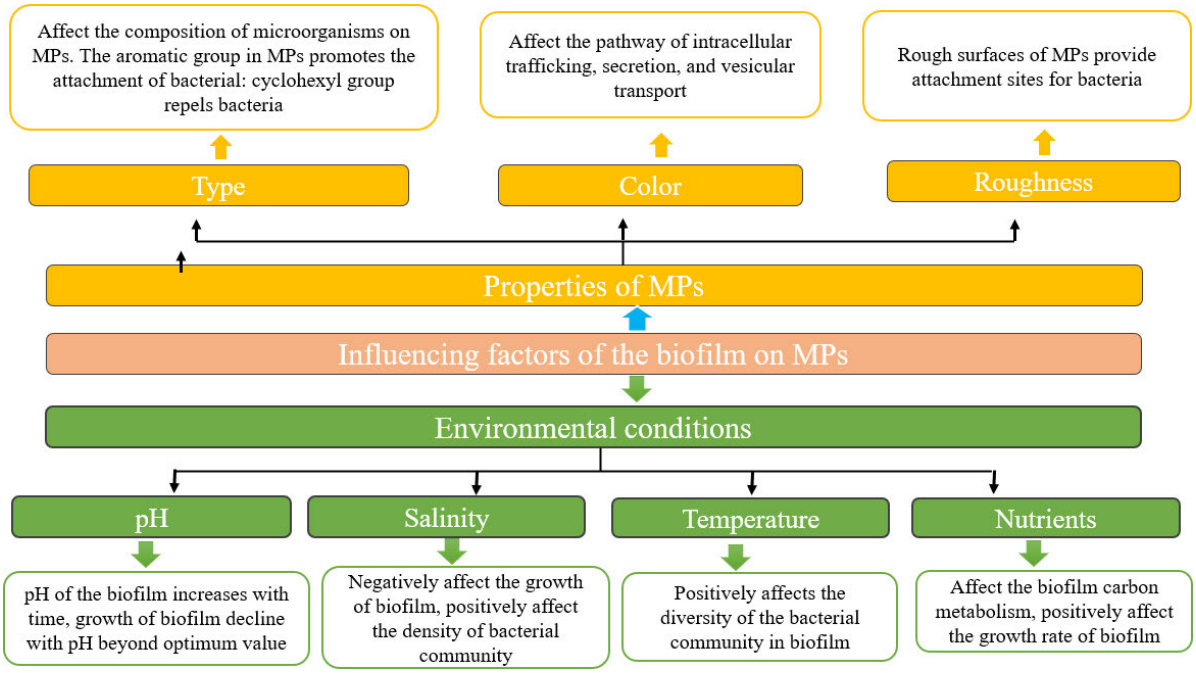


Figure 2. 4: The interaction of environmental factors and MP properties with biofilm (Wang *et al*., 2021b).

2.8 Antibiotic-resistant genes in Plastisphere

Microplastics have been discovered to provide new substrates for the development of biofilms in aquatic environments, with the potential to produce ARGs (Harrison *et al*., 2020, Kaur *et al*., 2021). Owing to their unique ecological and environmental effects on aquaculture, ecosystems, and people, MP-ARG interactions have drawn interest and transformed into research hotspots on a global scale. Drug-resistant bacteria have been shown to possess sulfonamide resistance genes, aminoglycoside resistance genes, beta-lactam resistance genes, and macrolide resistance genes, which persist and propagate in the aquatic environment, and biofilm is primarily involved in the accumulation of ARGs (Harrison *et al*., 2018, Li *et al*., 2020b). Microplastics are microbial pathogen reservoirs and a hotspot for HGT because they are colonized and encased by a variety of biofilm-forming microbial consortia along their lengthy journey from source to sink (Schluter *et al*., 2015, Wu *et al*., 2019b, Li *et al*., 2020b).

Researchers have recently investigated how MPs and contaminants interact to cause the growth of ARGs. In a comparative analysis of aquatic waste, Sucato *et al.,* (2021) found that there are a rising number of ARGs that cause antimicrobial resistance (AMR), including tetracyclines (*tetA* and *tetW*), ß-lactams (*blaTEM* and *blaCTXM*), erythromycin (*ermB*), quinolones (*qnrS*), and sulfonamides (*sulII*) with class 1 integrase. The ARGs profiling and the bacterial community makeup of biofilm-forming *Pseudomonas* are characterized as a critical host for ARGs in polyhydroxyalkanoates (PHA) and PET microplastic biofilms (Kaur *et al*., 2021) (Kaur et al., 2022). The ARGs (*sul1, sul2, tetA, tetO, tetW, Chl*, and *aac(6')-Ib*) of biofilm-forming bacteria (*Bacillus*, *Pseudomonas*, and *Mycobacterium*) colonizing PE and PP type microplastics increased in estuarine circumstances (Kaur *et al*., 2021). Research on PVC MPs from aerobic granular sludge systems has found significant AMR-inducing mechanisms, including the increased abundance of *intI1* and ARGs and efflux pumps, as well as polyphosphate accumulating organisms (POAs) such as *Chloroflexi*, *Actinobacteria*, *Bacteroidetes*, and *Nitrospirae* (Dai *et al*., 2020). ARGs and MGEs have been linked to Vibrio spp., which has been discovered to be a significant biofilm-forming pathogenic bacteria that colonizes MPs in estuarine water environments (Kaur *et al*., 2021). Various pathogens, including *Vibrio* species (Kirstein *et al*., 2016, Pedrotti *et al*., 2022, Leighton *et al.*, 2023), *Escherichia coli* (Gao *et al*., 2021, Yi *et al*., 2021), *Pseudomonas aeruginosa* (Wu *et al*., 2022a, Ayush *et al.*, 2022), and *Staphylococcus aureus*, are known to be associated with MPs, posing significant health risks. Eukaryotic pathogens such as fungi (*Candida* and *Aspergillus species*) (Bule Možar *et al*., 2023), algae (De-la-Torre, 2020, Wang *et al.*, 2023a), and protozoa (*Cryptosporidium and Giardia*) (Zhang *et al*., 2022a) also contribute to the complexity of MP-associated biofilms. Recent studies conducted in South Africa have explored the microbial communities associated with plastic debris (Zadjelovic *et al*., 2023, Joannard and Sanchez-Cid,

2024). These studies have identified various pathogens and ARGs within biofilms, raising concerns about their impact on public health and the environment (Table 2.3).

Table 2. 3: Summarized the major pathogens/ARGs reported in plastisphere communities, including both eukaryotes and prokaryotes

Category	**Pathogen**	**Description**	**Reference**
Prokaryotes	*Vibrio species*	Includes *Vibrio cholerae* (cholera), *Vibrio parahaemolyticus* (gastroenteritis), *Vibrio vulnificus*	(Kirstein *et al.*, 2016, Pedrotti *et al*., 2022, Leighton *et al.*, 2023)
	Escherichia coli	Pathogenic strains like *E. coli O157* cause severe foodborne illnesses	(Gao *et al*., 2021, Yi *et al*., 2021)
	Pseudomonas aeruginosa	Opportunistic pathogen known for antibiotic resistance and infections, especially in immunocompromised individuals	(Wu *et al.*, 2022a, Ayush *et al.*, 2022)
	Staphylococcus aureus	Includes MRSA, causing a variety of infections	(Imran *et al*., 2019, Pham *et al*., 2021)

Eukaryotes	Fungi	Pathogenic fungi like *Candida* species (candidiasis) and *Aspergillus* species (aspergillosis)	(Bule Možar *et al.*, 2023)
	Algae	Can produce harmful algal blooms, releasing toxins affecting aquatic life and human health	(De-la-Torre, 2020, Wang *et al*., 2023a)
	Protozoa	Protozoan pathogens like *Cryptosporidium* and *Giardia*, affect water quality and public health	(Zhang *et al*., 2022a)
Antibiotic Resistance Genes	ARGs	Horizontal gene transfer including ARGs is a significant concern in the plastisphere	(Xu *et al*., 2022, Yu *et al*., 2023)
South African studies	*Acinetobacter*	*aac(6')-Ib-cr* (Ciprofloxacin resistance)	Freshwater, South Africa; (Joannard and Sanchez-Cid, 2024)
	Klebsiella	*qnrB* (Ciprofloxacin resistance)	Riverine systems; (Zadjelovic *et al*.,

			2023, Joannard and Sanchez-Cid, 2024)
	Pseudomonas	*aac(6')-IIc* (Gentamicin resistance)	Riverine systems; (Joannard and Sanchez-Cid, 2024)

2.9 Isolation and identification of MPs from aquatic environments

Microplastics, or microscopic plastic particles less than 5 mm in size, are common pollutants in aquatic ecosystems. Their isolation and identification are critical for understanding their distribution, potential environmental consequences, and human health risks. There are several ways for isolating and identifying MPs, each with advantages and limits.

2.9.1 Filtration and Sieving

Filtration and sieving are the primary approaches for removing MPs from surface water and sediment samples. Surface water samples are typically passed through a series of stainless-steel sieves with decreasing mesh sizes, ranging from 5 mm to 20 µm (for the sieving method) while funnel, filter membrane, and vacuum pump system (for filtration method), to catch particles of varied sizes (Tirkey and Upadhyay, 2021). While sediment samples can be processed with a bigger sieve or filters. To reduce volume for isolation of MP particles, drying can be used if no visible plastic particles (<500 µm) are present. The collected material on the filters is then inspected under a microscope to separate MPs from organic and inorganic detritus. Previous studies demonstrated the effectiveness of filtration in isolating microplastics from environmental samples. Lindeque *et al.*, (2020) evaluated various size trawls (100, 333, and 500µm) to capture MPs from coastal waters. They highlighted that smaller pore-sized mesh

resulted in a collection of 2.5-fold and 10-fold greater MP concentrations than larger pore-sized meshes (333 and 500µm) (Lindeque *et al.*, 2020). Filtration or sieving is preferred because it is simple and efficient in handling vast amounts of water. However, it can be time-consuming, and the accuracy of particle identification is affected by the filter's pore size and the operator's experience.

2.9.2 Microscopy

The use of microscopy plays a role in identifying MPs. After isolating samples from the environment or a specific source researchers commonly utilize a microscope to observe and quantify MP particles by analyzing their shape, color, and size (Mai *et al.*, 2018). Advanced microscopy methods, such as scanning electron microscopy (SEM) provide high-resolution images that facilitate, in-depth morphological analysis (Alak *et al.*, 2022). Fluorescence microscopy is another method that can be employed when MPs are stained with fluorescent dyes to make them stand out more visible against organic materials. Observing MPs through microscopy provides evidence of their presence; however, it is often combined with other validation techniques since distinguishing plastic, from non-plastic particles solely based on appearance can pose challenges

2.9.3 Fourier transform infrared (FTIR)

FTIR spectroscopy is commonly used for the analysis of MPs in aquatic environments, by identifying their chemical properties through spectra patterns. The FTIR techniques include micro FTIR (µ-FTIR) and attenuated total reflection (ATR-FTIR) (Alak *et al.*, 2022). The use of the ATR-FTIR method the infrared light is directed at a sample to measure the absorbed wavelengths, which are linked to the materials' chemical bond vibrations. Different types of polymers can be distinguished by their individual FTIR spectrum that serves as a "fingerprint" for identification purposes in the field of materials, science, and analysis. To analyze MPs

effectively using FTIR methods like transmission or ATR they are often separated into filters or slides for examination. Incorporating microscopy into FTIR techniques through Micro FTIR enables researchers to focus light onto a very small sample area using microscope optics as described in the studies (Qiu *et al.*, 2016, Alak *et al*., 2022).

Both methods using FTIR can identify even very small particles (as few as micrometers) and accurately determine the types of polymers present. However, it requires specialized equipment and can be time-consuming when analyzing large numbers of samples (Lv *et al*., 2021). The effectiveness of FTIR in identifying MPs has been supported by studies using sequential isolation techniques.

2.9.4 Pyrolysis-Gas Chromatography-Mass Spectrometry (Pyro-GCMS)

Pyrolysis gas chromatography-mass spectrometry (Pyro-GC/MS) is an advanced analytical technology for identifying and quantifying MPs. This process involves heating isolated MP materials to high temperatures (pyrolysis) to break them down into smaller molecules (Prasad *et al*., 2023). The pyrolysis products are then separated by gas chromatography and identified by mass spectrometry. This approach gives extensive information on polymer composition and can detect additives and pollutants found in MPs (Vairaperumal *et al*., 2024). Research conducted by Lou *et al*. (2022) focused on the accurate identification and quantification of MP using Pyro-GC/MS. The study analyzed five common MP materials (PE, PP, PS, PVC, and PMMA), revealing significant interactions during co-pyrolysis that affected quantitative calculations. To address this, new, more reliable indicators were proposed, significantly reducing quantification uncertainties for PE, PP, and PS. This study provides a valuable reference for enhancing the accuracy of mixed MP analysis (Lou *et al*., 2022). Additionally, a study conducted by Roscher *et al.* (2022) investigated the effluent of two German WWTP over one year to understand the temporal input of MPs into river systems. Monthly data were collected using FTIR spectroscopy and Pyro-GC/MS. Results demonstrated a homogeneous

polymer composition dominated by polyolefins, with elevated MP concentrations during winter months influenced by heavy rainfall and turbidity. This study highlights the importance of assessing background parameters in MP monitoring and provides valuable temporal data for future risk assessments (Roscher *et al.*, 2022). The Pyro-GCMS is one of the most sensitive and specific tools for analyzing MPs (Tirkey and Upadhyay, 2021, Zhang *et al*., 2023). However, it is also one of the most complicated and costly procedures, necessitating substantial sample preparation and technical knowledge.

2.10 Identification of biofilm associated with microplastics

Biofilms found on MPs consist of a complex community of bacteria, archaea, fungi, and viruses embedded within a self-produced extracellular polymeric substance (EPS) (He *et al*., 2022b, Yan *et al.*, 2024). The unique physical and chemical properties of MPs, such as hydrophobicity and surface area, provide a favourable environment for microbial colonization, allowing biofilms to persist in various environmental conditions (Qin *et al*., 2023). Furthermore, biofilms on MPs facilitate the transport of microorganisms across ecosystems, contributing to the spread of pollutants, pathogens, and ARGs (Yan *et al*., 2024).

Given the growing concerns about MP pollution and its association with biofilms, researchers increasingly employ advanced molecular techniques, including metagenomic sequencing, to investigate the microbial communities inhabiting these surfaces (He *et al*., 2022b). This approach provides a comprehensive insight into the taxonomic and functional diversity of biofilms on MPs, bypassing the limitations of culture-dependent methods (Panaiotov *et al.*, 2021).

2.10.1 Sample collection and biofilm isolation

Biofilm sampling from MPs typically involves the collection of plastic debris from aquatic environments, including oceans, rivers, and WWTPs. Microplastic samples are retrieved using various methods, such as manta trawls, plankton nets and stainless-steel sieves for surface waters (for flowing MPs) or sediment grabs for bottom environments (Campanale *et al*., 2020, Zheng *et al.*, 2021). Once collected, the samples are transported under sterile conditions to prevent contamination. Furthermore, once the MPs are rinsed with sterile water to remove free-floating microorganisms, leaving behind the more tightly adhered biofilms. In some cases, biofilms are removed from the surface of MPs using mechanical or chemical methods, such as ultrasonication or enzyme digestion, for further analysis (Li *et al.*, 2023).

2.10.2 Microscopic identification of biofilms

Microscopic techniques are commonly employed as the first step to visualize biofilm structure on MPs. Scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) are frequently used to assess biofilm thickness, surface coverage, and microbial morphology on MP surfaces (Tu *et al*., 2021, Pawano *et al*., 2024). The SEM allows researchers to observe the surface morphology and microbial communities at high magnifications, providing insights into the physical attachment of microorganisms to the plastic surface (Ramsperger *et al.*, 2020, Tu *et al.*, 2021, Pawano *et al*., 2024). For example, a study by Ramsperger *et al.* (2020) used SEM to examine the early stage of biofilm formation on PA, PET, and PVC after incubation in freshwater and artificial seawater, highlighting the widespread nature of biofilm formation on common plastic types (Ramsperger *et al*., 2020).

2.10.3 Metagenomic sequencing for biofilm identification

Metagenomic sequencing is a powerful tool for identifying the microbial communities within biofilms on MPs (Shen *et al.*, 2023). Unlike traditional culture-based methods, which are limited by the ability to grow only a fraction of environmental microbes, metagenomics allows for the culture-independent characterization of the entire microbial community present in a sample (Tyagi *et al.*, 2024). This includes the identification of both culturable and non-culturable microorganisms as well as functional genes associated with pathogenicity and antibiotic resistance (Imchen *et al.*, 2022, Shen *et al.*, 2023).

In the metagenomic workflow, DNA is extracted from the biofilm on MPs and subjected to high-throughput sequencing platforms such as Illumina MiSeq or NovaSeq (Giangeri *et al*., 2022, Pawano *et al*., 2024). The resulting sequence reads are then analyzed using bioinformatics tools to identify taxonomic groups and functional genes (Miao *et al.*, 2022). Tools such as Kraken2, MetaPhlAn, and QIIME2 are commonly employed for taxonomic classification, while functional annotations can be performed using databases such as the Kyoto Encyclopedia of Genes and Genomes (KEGG) or the Comprehensive Antibiotic Resistance Database (CARD) (Malla *et al*., 2023b, Espinoza *et al.*, 2024). For example, a recent study by Qian *et al.* (2024) used metagenomic sequencing to investigate biofilm communities on MPs in the Dongshan Bay coast. They found that the dominant phyla on biofilm of PS were *Proteobacteria* (93.43%), *Planktomycetes* (1.38%), and *Bacteroidetes* (1.21%), biofilm on PET was dominated by *Proteobacteria* (65.95%), *Chloroflexi* (6.42%), *Actinobacteria* (5.54%), *Firmicutes* (2.16%), *Planctomycetes* (2.12%), *Bacteroidetes* (1.35%), and *Nitrospirae* (1.13%) (Qian *et al.*, 2024).

The shotgun metagenomic method sequences the total DNA in a sample, allowing for the identification of all microbial taxa (bacteria, archaea, viruses, fungi) and functional genes present in the biofilm (He *et al*., 2022b). Shotgun metagenomics provides a comprehensive

picture of both microbial diversity and the functional capacity of the biofilm, making it particularly useful for the identification of ARGs and virulence factors (Pawano *et al.*, 2024). However, it requires more computational resources and cost compared to amplicon sequencing. While 16S rRNA amplicon sequencing technique targets the 16S ribosomal RNA gene, a conserved gene found in bacteria and archaea, to identify the taxonomic composition of microbial communities (Tora *et al.*, 2022). While it provides high-resolution taxonomic data on bacteria and archaea, it does not offer insights into the functional genes or the presence of viruses and fungi in biofilms (Wang *et al.*, 2021b, He *et al*., 2022b). Nevertheless, amplicon sequencing is more cost-effective and widely used for microbial ecology studies. Tora *et al.* (2022) conducted a study using 16S rRNA sequencing to characterize microbial communities on PP and PE from the North Atlantic and Great Garbage Patches. The study highlighted significant differences between the microbial communities in the two ocean regions, with *Proteobacteria*, *Cyanobacteria* and *Firmicutes* as dominant biofilm-forming taxa (Tora *et al.*, 2022). Similarly, Rohrbach *et al.* (2022) used 16S rRNA gene amplicon sequencing to examine microbial communities colonizing various MP polymers, such as PE, PP, PS, PA, and PET, in terrestrial landfill soils over 14 months. The research identified dominant taxa including *Chloroflexota* and *Gammaproteobacteria*, with enrichment of potential pathogens like *Aeromonas hydrophila* and *Pseudomonas aeruginosa*. The study demonstrated that MP polymer type significantly shaped biofilm formation and microbial succession, with polymers like PA and PET exhibiting higher biofilm densities and supporting diverse microbial communities (Rohrbach *et al.*, 2023). Additionally, the study revealed that MPs influenced greenhouse gas metabolism, highlighting their potential role in driving methane emissions in terrestrial environments.

Metagenomic sequencing also enables the identification of functional genes within the biofilm community. Using functional gene annotation pipelines, researchers can identify genes

involved in biofilm formation, pathogenicity, and antibiotic resistance (Imchen *et al.*, 2022, Messer *et al.*, 2024). For example, ARGs associated with tetracycline, sulfonamide, and macrolide resistance are commonly found in biofilms on MP, particularly in environments impacted by anthropogenic pollution, such as wastewater treatment plants and agricultural runoff (Wu *et al.*, 2022b).

CHAPTER III

3.0 IMPACT OF DIFFERENT POLLUTION SOURCES ON WATER QUALITY, MICROPLASTIC ABUNDANCE

3.1 Introduction

The rising concern of MP pollution in freshwater systems has led to extensive studies focusing on their occurrence, types, and potential impact on aquatic environments. In riverine ecosystems, MPs are commonly detected in various forms, including fibers, fragments, films, and beads, with polymers such as PE, PP, PS, and PET frequently identified (Esterhuizen and Kim, 2022). In South Africa, MP research has gained attention due to the country's reliance on rivers for domestic, agricultural, and industrial purposes. Studies from South Africa have highlighted the presence of MPs in several rivers, with fibers being the most prevalent type detected in surface waters. For example, research conducted on the Vaal River and Buffalo River demonstrated the widespread contamination by synthetic fibers, microbeads, and fragments originating from industrial, urban, and agricultural pollution sources (Bouwman *et al*., 2018, Nel *et al*., 2021).

Despite these efforts, research gaps persist, particularly in understanding how different pollution sources influence the type and concentration of MPs. Existing studies have primarily focused on MP abundance and distribution, but few have assessed the direct correlation between pollution sources such as WWTP effluents, agricultural runoff, and industrial

discharge and the specific types of MPs present in river systems (Horton *et al*., 2017, Chen *et al*., 2021). This lack of targeted investigation hinders our ability to fully understand the pathways through which MPs enter freshwater systems and their broader environmental impact, particularly in regions with complex pollution profiles like South Africa.

The uMsunduzi River is an important tributary of the Umgeni River between Nagle and Inanda dams, and it flows out into the Indian Ocean at Durban (Gemmell and Schmidt, 2013, Shozi, 2015). The river is a source of water for domestic, agricultural, and industrial purposes in the uMsunduzi municipality. The river is impacted by different activities that contribute to increased pollution levels, including the introduction, of raw sewage spills, furnace oil leaks, and chemical contaminants from sources such as urban runoff, industrial discharge, agricultural practices, and wastewater effluents (Matongo *et al*., 2015). Several water management studies have been conducted on the uMsunduzi River, including research on heavy metals, pharmaceutical contamination of surface water, and potential risks associated with its domestic, recreational, and agricultural uses (Matongo *et al.,* 2015). However, no studies have been conducted to assess the impact of different pollution sources on the polymer types of the river. The purpose of this study is to determine the influence of different pollution sources (such as treated effluents from wastewater treatment plants (WWTP), runoff from agricultural areas (AA), urban area (UA), and industrial area (IA) on the water quality, type, and number of MP particles in the uMsunduzi River, located in KwaZulu-Natal (KZN) province.

3.2 Materials and Method

3.2.1 Study area

The uMsunduzi River in KwaZulu-Natal was selected as the study area. The river flows through highly industrialized areas, and receives runoff from the agricultural sector, effluent from wastewater treatment plants, runoff from rural and urban communities. Additionally, the uMsunduzi River is also a key tributary of the uMngeni River, contributing significantly to the larger river system and impacting water quality downstream. The exact locations of the sampling sites were selected to purposely represent different pollution sources: Site 1 (site near the urban area, UA-impacted site), Site 4 (site near the industrial area, IA-impacted site), site 3 (site near agricultural sector, AA-impacted site), Site 2 (downstream of wastewater treatment plant effluent, WWTP-impacted site). A map of sampling sites is shown in (Figure 3.1). A global positioning system (GPS) was used to verify the location of each sampling site. Table 3.1 represents site coordinates, activities, and physicochemical parameters.

Table 3 1: Geographical position and observed possible activities of each sampling site at the uMsunduzi River.

Site	Name of sites	Coordinates	Sample collected	Potential activities
UA impacted site	FNB	29°36'43.2"S 30°23'13.8"E	Surface water	Runoff from Urban communities
WWTP impacted site	Pine tree	29°35'49.3"S 30°26'21.3"E	Surface water and sediment	Downstream of the wastewater treatment plant
AA impacted site	Braai takeout	29º36’29.3”S 30º27’09.0”E	Surface water and sediment	Runoff of agricultural sector and rural communities
IA impacted site	Low level	29°36'07.9"S 30°24'48.5"E	Surface water	Industrial waste

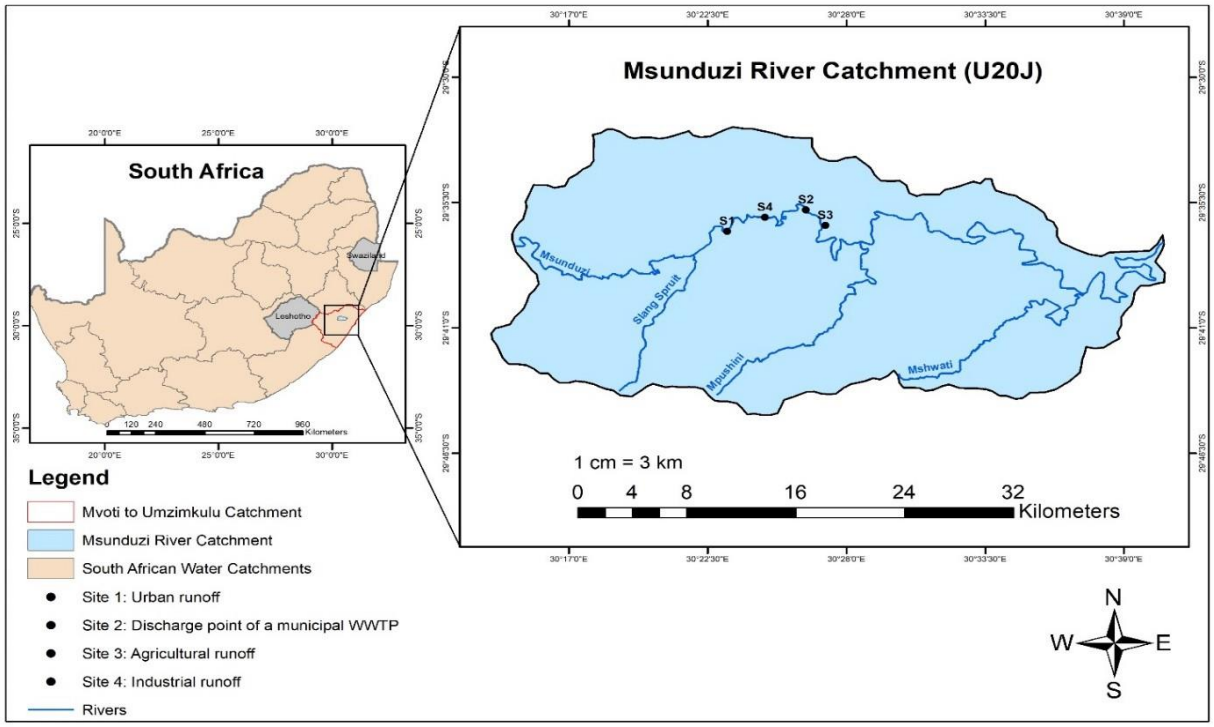


Figure 3. 1: Geographical location of UMsunduzi River in KwaZulu Natal and sampling sites along the catchment.

3.2.2 Sample collection

Surface water and sediment samples were collected from the 4 sites (UA, WWTP, AA, and IA) with geographical coordinates and possible activities as listed in (Table 3.1). Surface water samples were collected from all sites (UA, AA, WWTP, and IA), while sediment samples (AA and WWTP) could only be obtained from two accessible locations. Composite sampling was performed at each site for both surface water (12 samples) and sediment (6 samples) by collecting multiple subsamples (at 2 to 5-minute intervals) from the flowing river over a period of time. These subsamples were then combined to provide a more comprehensive representation of the number of MP per liter, ensuring that temporal variations in the flow were accounted for. The samples were collected in liter bottles and were promptly transported to the laboratory on ice and were further

filtered through 0.5 mm, 0.18 mm, 0.1 mm, and 0.025-mm steel mesh size sieves (Hidalgo-Ruz *et al.*, 2012).

3.2.3 Physicochemical analysis of water samples

The physicochemical parameters of the surface water such as temperature, pH, dissolved oxygen (DO, mg/l), total dissolved solids (TDS), specific conductivity (µS/cm), and salinity, were measured on-site using YSI 556 multiprobe (Yellow Spring Instrument California). Water samples were further analyzed in the laboratory for the COD Digestion VIAL, LR HW PK/150 was used to analyze COD.

3.2.4 Isolation of Microplastics

In this study, the procedure outlined by Hossain *et al* (2023) was used to isolate the MPs from both surface water and sediment samples. Each sample was collected and filtered through the sieves, the residues were then washed off with 50 ml distilled water (dH_2O) into a beaker and allowed to dry in the oven at 90 °C overnight. The organic components were removed by adding 50 ml (30% hydrogen peroxide (H_2O_2) to the dried samples (Nuelle *et al.*, 2014). The mixture was heated and stirred at 75^oC until dry. The MPs were separated by performing the density separation method using sodium chloride (NaCl) solution with a density of 1.2 g cm^{-3} (Hidalgo- Ruz *et al.*, 2012). Materials with a density above 1.2 g cm^{-3} settled to the bottom of the conical flask and the floating materials (MP particles) were carefully transferred into fresh conical flasks. All the light materials (MP particles) were filtered through a 20 µm mesh size sieve and rinsed with 50 mL dH_2O and then washed off into a clean beaker with 50 mL dH_2O. The materials were thereafter filtered utilizing a 1.22 µm pore size glass fiber filter paper using a filtration unit (vacuum system). Subsequently, the filter was placed in a clean Petri dish and allowed to dry in the oven at 60^oC.

3.2.5 Characterization of MPs in surface water and sediment samples

3.2.5.1 Visual characterization

A light microscope with magnification of 4x (Nikon, Y-TV55 microscope) was used to identify and quantify the MP particles on the filter paper. The high-resolution images were captured using MoticamBTW camera (SSID: MCX_BTW_1462, Motic China group) connected to the microscope. Morphological characterization of the MPs was performed based on the shape, sizes, and color (Hidalgo-Ruz *et al.*, 2012, Hossain *et al.*, 2019). The observed MPs are classified into three types based on their shape: fiber, pellet, and fragments (Nie *et al.*, 2019).

Fiber is a long and thin line with a slender shape that comes in different colors (transparent, red, blue, dark blue, etc.). The fragment was characterized as a piece of debris. The film appears in the shape of broken pieces or slices. The pellets were considered a dimensional sphere (Hossain *et al.,* 2019). The MP sizes were estimated based on the pore sizes of the mesh used to isolate them. The abundance of MPs per liter (particles/L) was the unit used for MPs in surface water.

3.2.5.2 Chemical characterization

3.2.5.2.1 FTIR

The MPs on the filter papers were then dried to eliminate moisture, which could interfere with infrared analysis. The chemical composition of each piece of plastic debris on the filter paper collected was identified using the attenuated total reflectance Fourier Transform Infrared (ATR-FTIR) spectroscopy using Perkin Elmer, Spectrum two with ATR. Wavelength spectra were recorded in the mid-infrared range (4000-400 cm^{-1}) and at resolution 4 cm^{-1}. The plastic characterization was performed by looking at the absorption bands (AB) (Table 3.2) of samples with those in the published literature (Coates, 2000, Khan *et al.*, 2018) based on the infrared spectrum.

Table 3. 2: Range in wavenumber (cm^{-1}) with the regions

Range of wavenumber (cm^{-1})	**Regions**
2500-4000 cm^{-1}	Single bond region (e.g., O-H, N-H, and C-H)
2000-2500 cm^{-1}	Triple bong region (e.g., C≡C, and C≡N)
1500-2000 cm^{-1}	Double bond region (e.g., C=C, and C=O)

600-1500 cm^{-1}	The fingerprint region. The region from 1000 to 1500 cm^{-1} some classify it for C-O and C-C and other bending vibrations. While region 400-700 cm^{-1} classify it as a fingerprint region.

3.2.5.2.2 Py GC-MS analysis

For the Pyro-GC/MS analyses, MP particles were collected on the filter papers and were first viewed under a Stereo Olympus microscope with a Euromex fiber optic light source (EK-1) to visually identify the MPs. These identified microplastic particles were then used for polymer composition analysis using Pyro-GC/MS. The samples were then pyrolyzed in a multi-shot pyrolyzer, EGA/PY-3030 D, (Frontier Lab, Japan) attached to an ultra-alloy capillary column (30 m x 0.25 mm, 0.25 μm). Approximately 100 to 150 μg of the sample were pyrolyzed at 550°C for 20 seconds and the interface temperature to the analytical column was set at 350°C. The chromatographic separation of the pyrolysis products was performed using an ultra-alloy capillary column (Frontier Lab, Japan) (30 m × 0.25 mm, 0.25 μm). The injection temperature was set to 280°C and the column flow rate was set to 1.0 mL/min with helium used as a carrier gas. The GC temperature programme used was: (i) hold at 50°C for 2 min; (ii) ramp from 50°C to 200°C at a rate of 3°C/min; (iii) then hold for a further 4 min. The ion source and interface temperatures in the mass spectrometer were set to 200°C and 300°C, respectively. The scan range used for the mass selective detector was from m/z 40-650. The pyrolysis products were identified by comparing their mass spectra with the mass spectra in a library database (NIST14 and WILEY10).

3.3 Results

3.3.1 Physicochemical quality of uMsunduzi River.

The physicochemical parameters recorded at each site included temperature, pH, and DO concentration (reported in mg/L), along with salinity, TDS, and specific conductivity for the uMsunduzi River (Table 6). The average temperature was 19ºC, with values ranging from 18ºC to 20ºC. The pH varied from 7.03 to 7.51 (Table 3.3), with an average pH value of 7.38, depicting the optimum pH for river water (neutral). The specific conductivity values varied from 143.5 to 281 µS/cm which is also within the acceptable range. The TDS concentration of collected water samples in four sites ranged from 127.25 to 181.5 mg/l. The DO concentration in the water column ranged from 2.3 to 4.01 mg/L, whereby the UA-impacted site showed the lowest DO concentration. The COD concentration in this study varied between 89.67 to 108.20 mg/L (Table 3.3). The average COD values for the sampling sites the highest was observed in the IA site at 108.33 mg/L, followed by the WWTP-impacted site at 105.33 mg/L, the UA site at 104.68 mg/L, and the lowest was in the AA impacted site at 89.67 mg/L.

Table 3. 3: Physicochemical parameters of sampling sites from uMsunduzi River.

	Surface water samples				**Standards**
	UA	**WWTP**	**AA**	**IA**	
Temperature (ºC)	18	19	20	19	20-30 [a, c, d], 25 [b, d]
DO (mg/l)	2.8	3.3	2.3	4.01	4-6[a], 6 [b, c]
pH	7.22	7.37	7.43	7.51	6.5-8.5 [a, b, c, d]
Salinity	0.13	0.14	0.11	0,10	
TDS (mg/L)	127.25	181.50	149.13	130.25	1000 [a]

Specific conductivity (µS/cm)	251.6	281,0	143.5	196.25	700 [a], 1000 [b]
COD (mg/L)	104.67	105.33	89.67	108.20	4 [a,c], <30[d]

"a"," b", "c", "d", (EPA, 2006, Water, 2012), (Li, 2016), (Organization, 2017, Organization, 2020) , South African Department of Water Affairs(Affairs and Forestry, 1993, Holmes, 1995) respectively.

3.3.2 Comparing microplastic abundance in surface water and sediment

The analysis revealed the pervasive presence of MPs across all sampled locations, both in surface water and sediment. This consistent detection underscores the widespread nature of MP pollution in the studied rivers, highlighting the urgent need for further investigation and remediation efforts. Through observation, the number of MP particles in surface water ranged from 49 to 69 MP particles/L. The results illustrated a high number of MPs per liter of surface water was recorded at the IA-impacted site with an abundance of (69 particles/L); this site is impacted by industrial activities, as shown in (Figure 3.2). The second highest concentration of MP particles in the uMsunduzi River was recorded in the WWTP-impacted site (51 particles/L) impacted by wastewater treatment plant effluent, followed by the UA site (49 particles/L) impacted by urban activities, and then the AA-impacted site (39 particles/L) impacted by agricultural sectors.

It was observed that the sediment samples from the river had a number of MP above that of the surface water for both WWTP impacted site (sediment: 52 particles/L; surface water: 51 particles/L) and AA impacted site (sediment: 87 particles/L; surface water: 39 particle/L) sites. It must be noted that sediment samples were taken from only two sites (WWTP and AA impacted sites), in the uMsunduzi River due to accessibility.

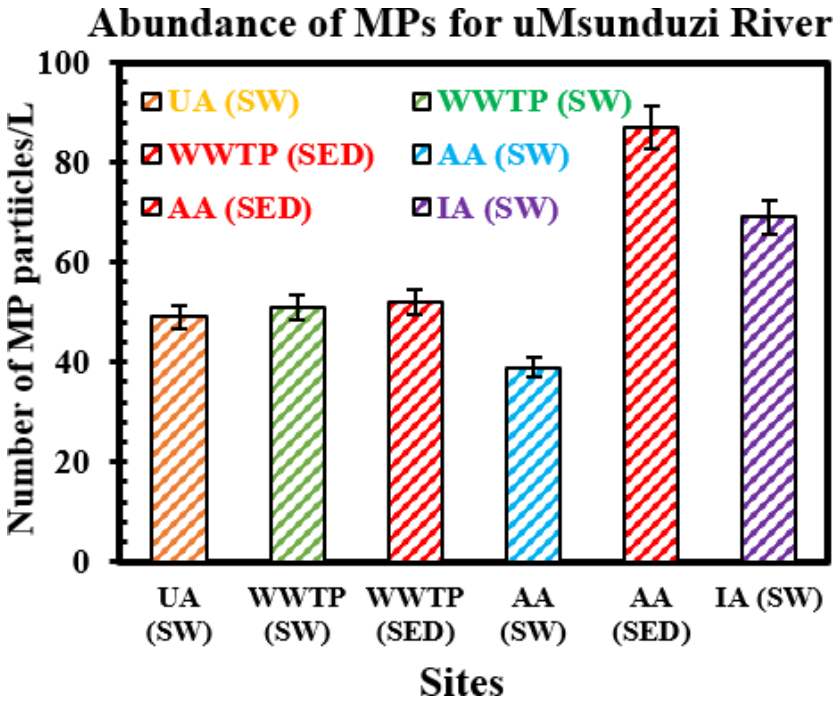


Figure 3. 2: Total number of MPs found in all four sampling sites from surface water and sediment samples in the uMsunduzi River.

3.3.3 Characterization of microplastic

3.3.3.1 Shape distribution of microplastics: Fibers, Fragments, and Pellets of MPs

The analysis of MP samples from uMsunduzi River revealed a diverse array of particle shapes, including fibers, fragments, and pellets in both surface water and sediment samples. There were four different colors of MPs observed including blue, transparent, dark blue, and red (Figure 3.3B). Fibers were the most dominant shape accounting for 60% and 59% of the total MPs observed in surface water and sediment samples, respectively (Figure 3.3A). In the UA-impacted site (surface water), fibers were particularly abundant, with 37 particles/L. Similarly, fibers were present in high numbers at the WWTP-impacted site with 30 particles/L in surface water and 32 particles/L in sediment samples. Also, the AA-impacted site with 26 particles/L in surface water and 36 particles/L in sediment samples, while the IA-impacted site had the highest fiber abundance in surface water, with 42 particles/L. Interestingly, fragments were the least common MP type for surface water and sediment samples, both accounting for 4%.

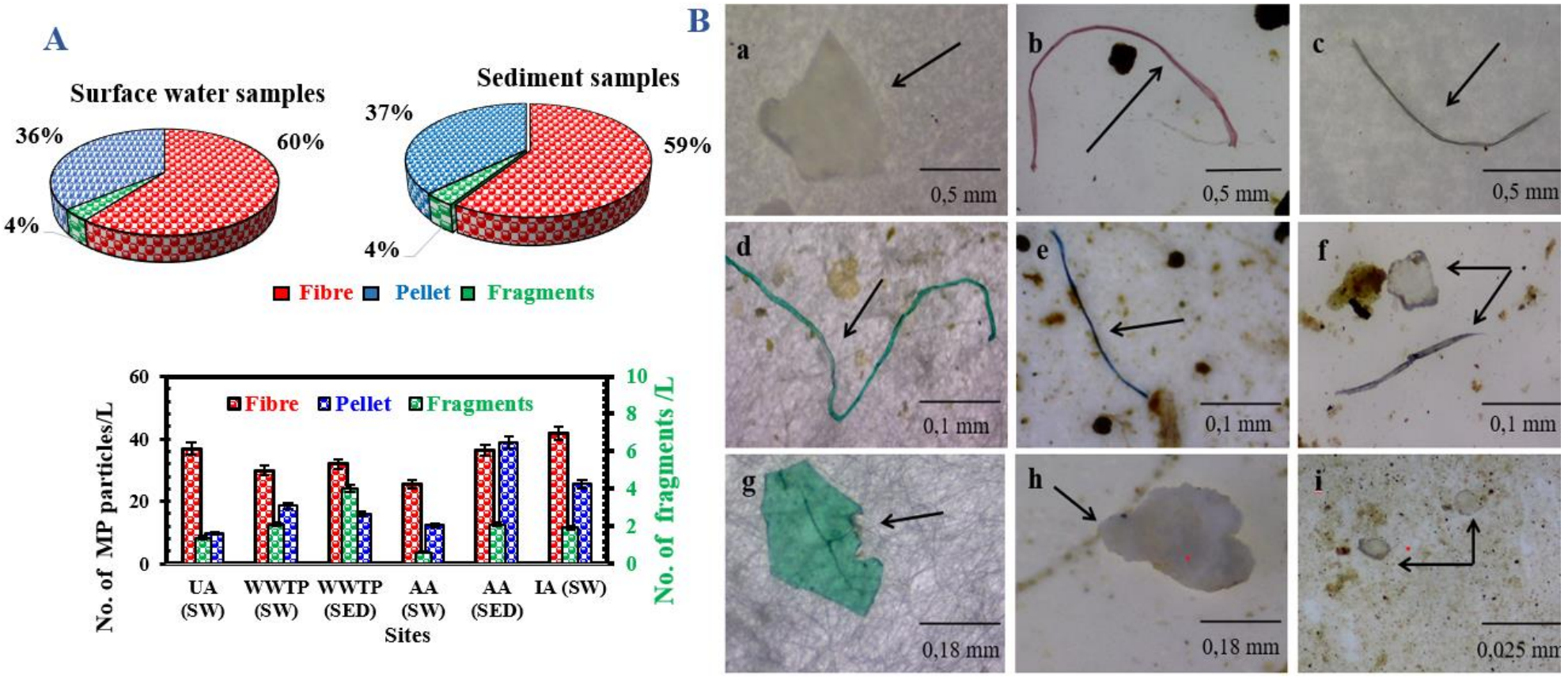


Figure 3. 3: Shape distribution of microplastic particles and percentage form distribution in uMsunduzi River (A). Images of different shapes of MPs in uMsunduzi River (B) The arrow indicates fragment (a), fibers (b-e), pellet and fiber (f), fragments (g), foam (h), and pellet (i).

The size variation of MPs in the uMsunduzi River was categorized into four sizes using steel mesh sieves: 0.5 mm, 0.18 mm, 0.1 mm, and 0.025 mm (Figure 3.4). These size results are presented in terms of the percentage distribution of particles in each stage to determine and explain the distribution more accurately. The UA-impacted site in surface water, the largest size (0.5 mm), dominated, accounting for 40% of the total number of MPs, followed by 0.18 mm (23%), 0.025 mm (20%), and then 0.1 mm (17%). In the WWTP-impacted site surface water sample, the 0.1 mm size was most prevalent contributing to 34% of the total number of MPs, while in the WWTP site sediment sample, the smallest size particles (0.025 mm) were more dominant, making up 35% of the number of MPs. In the AA-impacted site, the surface water sample showed the highest number of MPs in the 0,5 mm size (36%), and 0.025 mm size showed the lowest number of MPs 17%. However, sediment samples from the AA-impacted site showed 30% of MPs for the 0.18 mm size particles, and the smallest size particles (0.025 mm) accounting for 26%. Finally, in the IA-impacted site, surface water sample had a distribution of MP sizes, with 34% in the 0.5 mm size and 29% in the 0.18 mm size, while the smaller MP sizes (0.1 mm and 0.025 mm) were present but less dominant, each contributing about 18% of the total.

The Spearman correlation test was run to determine the relationship between MP sizes and abundance in surface water and sediment samples across the sites. The surface water (uMsunduzi River) for UA-impacted site demonstrated a strong statistically significant correlation with r = 0.9509 and p = 0.0491 whereas the other sites (WWTP, AA, and IA-impacted sites) showed insignificant correlation with p-value > 0.05 (Table S5). Whereas in the sediment samples, WWTP-impacted site indicated almost no correlation (r = -0.05499) while the AA-impacted site demonstrated a moderate negative correlation (r = -0.6833).

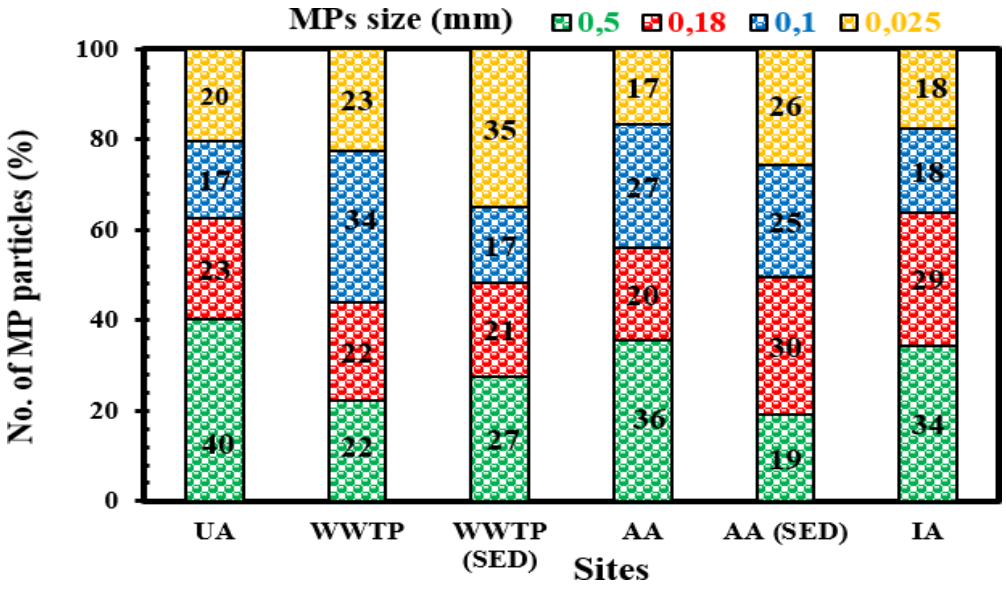


Figure 3. 4: Size distribution for surface water and sediment samples in uMsunduzi River.

3.3.4 The distribution, Micrograph, and chemical characterization of MPs

3.3.4.1 MPs identified using ATR-FT-IR analysis.

In the uMsunduzi River, the polymer types identified through the FTIR analysis vary across different sites, reflecting the pollution sources (Figure 3.5). The spectral band in the UA-impacted site sample (surface water) near 1002 cm^{-1} is typically associated with C-C stretching vibrations in the aliphatic compound, which could suggest the presence of long-chain aliphatic hydrocarbons, such as those found in PE. While the spectral band 1509 cm^{-1} corresponds to C=C bending or aromatic skeletal vibrations in aromatic compounds. This points to PS, which contains aromatic rings as part of its structure, making it a possible polymer type present. Another intense peak near 1702 cm^{-1} is indicative of C=O stretching, which is characteristic of carbonyl compounds like esters or aldehydes. This is a characteristic of PET, a polymer that has ester functional groups in its backbone. Therefore, based on the spectral analysis, the MPs detected in the UA site may include PE, PS, and PET.

The spectral band in the WWTP and IA impacted site samples show similar peaks near 1509 cm^{-1}, which is typically associated with C=C stretching vibrations in aromatic compounds, suggesting the presence of a conjugated system, which is typical of PS. Spectral band near 1702 cm^{-1} is typically associated with C=O stretching vibrations, indicative of carbonyl groups. This is characteristic of PET, a polymer with ester bonds in its structure, which also contains carbonyl groups. The WWTP-impacted site sample also showed the presence of a C-C stretching peak at 1000 cm^{-1}, suggesting the presence of PP. The polymers likely present in the WWTP and IA-impacted site samples include PS, PP, and PET.

The FTIR spectra of the AA-impacted site sample showed intense peaks near 1002 cm^{-1}, 1702 cm^{-1}, and 3750 cm^{-1}. The peak at approximately 1002 cm^{-1} is typically associated with C-H bending vibrations suggesting the presence of PE. The peak around 1509 cm^{-1} corresponds to C=C stretching vibrations and is linked to aromatic compounds, indicating the presence of PS. The peak near 1702 cm^{-1} can be attributed to the carbonyl (C=O) stretching of aldehydes, characteristic of PET. The peak at 3750 cm^{-1}, which may indicate the presence of hydroxyl groups (-OH), suggests the potential presence of PVA or other hydroxyl-containing polymers. The C-C stretching peak at 1000 cm^{-1} suggests the presence of PP. Therefore, the polymers likely present in the AA-impacted sample include PE, PS, PET, and PVA.

The analysis of sediment samples from AA and WWTP-impacted sites of the uMsunduzi River showed significant peaks corresponding to various functional groups. The common spectral band in the WWTP and AA-impacted sites near 1092 cm^{-1} is typically related to the C-H aromatic in-plane bend characteristic of PC. A spectral band near 470 cm^{-1} indicates out-of-plane bending vibrations, possibly associated with alkyl chains or certain halogenated compounds. A spectral band near 2340 cm^{-1} typically represents the C≡C stretching vibrations or may indicate the presence of carbon dioxide (CO_2) if it's a broader band.

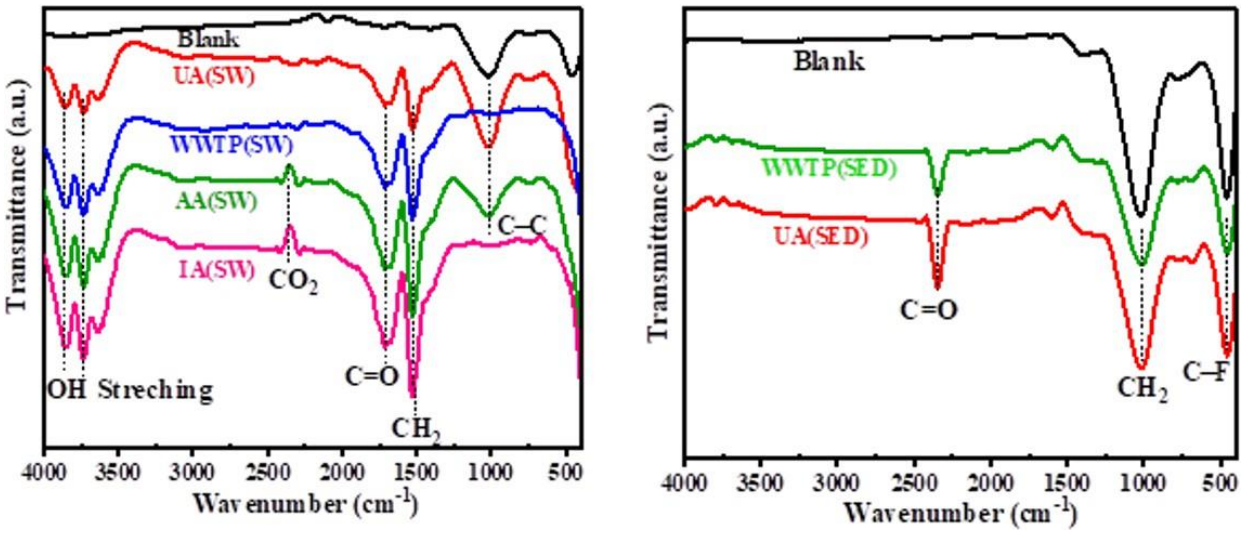


Figure 3. 5: ATR-FTIR analysis uMsunduzi River (A) surface water samples, (B) sediment samples.

3.3.4.2 MPs analysis using Py GC-MS

In the analysis of pyrolyzate compounds across various sites, distinct chemical profiles were observed, indicative of their probable polymer origins (Table 3.4). Characteristic products with relatively high yields were selected according to the pyrolysis law of plastics. At the UA-impacted site, 1,30-Triacontanediol was the dominant compound, with a spectra peak area of 40.96% and a retention time of 33.856 minutes, likely produced from PE. Heptacosane, detected at a 31.26% peak area and 42.12 minutes, and Nonacos-1-ene, with an 18.43% peak area at 38.783 minutes, both suggest origins from PE and nylon. At the WWTP-impacted site, the analysis revealed Benzene with a prominent 60.33% peak area at 2.285 minutes, pointing to PS as the source. Other compounds such as Propyphenazone (8.18%, 15.235 minutes) suggest pharmadrugs, Benzene, ethenyl- (5.13%, 5.984 minutes) suggest PS, and Benzaldehyde (3.78%, 7.943 minutes) further support PS, PP, PE, and nylon as the primary source. The AA-impacted site featured Benzene, ethenyl- at 25.15% peak area and 6.004 minutes suggest PS, 2,4-Hexadiyne (24.78%, 2.282 minutes), which suggests origins from ABS plastic, cellulose acetate plastic. Benzene, methyl- (6.54%, 3.453 minutes) confirmed PS, while Benzaldehyde (5.26%, 7.943 minutes) further confirmed the presence of PP, PE, PS, and nylon. At the IA-impacted site, Tetratetracontane was identified with a spectra peak area of 9.94% at 29.56 minutes, Hexacosane at 9.22% and 31.554 minutes, 1-Hexanol, 2-ethyl- at 5.09% and 9.916 minutes, and 1-Heptadecene at 4.5% and 31.786 minutes, all suggesting PE as the source. In the sediments at the WWTP-impacted site, Heneicosyl heptafluorobutyrate showed a substantial peak area of 72.78% at 41.09 minutes, indicating Polytetrafluoroethylene (PTFE), while 1,19-Eicosadiene at 23.32% and 48.681 minutes pointed to PE. Sediment analysis at the AA-impacted site revealed Hexacosane (59.27%, 32.724 minutes), Octacosane

(27.43%, 32.27 minutes), and Tetratetracontane (9.7%, 31.52 minutes), all correlating with PE as the likely source.

Table 3. 4: Characteristic pyrolyzate compounds, and respective spectra peak area (%), molecular structure, and probably produced from.

Pyrolyzate compounds	**Spectra peak area (%)**	**Retention time (min)**	**Chemical formula; PubChem CID**	**Probable produced from**	**References**
			UA (SW)		
1,30-Triacontanediol	40.96	33.856	$C_{30}H_{62}O_2$; 543982	PE	(Šunta *et al.*, 2021)
Heptacosane	31.26	42.12	$C_{27}H_{56}$; 11636	PE (HDPE/LDPE), Nylon	(Jaafar et al., 2022)
Nonacos-1-ene	18.43	38.783	$C_{29}H_{58}$; 156989	PE, Nylon	(Leslie *et al.*, 2022)
			WWTP (SW)		
Benzene	60.33	2.285	C_6H_6; 241	PS	(Lou *et al.*, 2022)
Propyphenazone	8.18	15.235	$C_{14}H_{18}N_2O$; 3778	Pharmadrugs	(Phong *et al.*, 2024)
Benzene, ethenyl-	5.13	5.984	C_8H_8; 7501	PS	(Lou *et al.*, 2022, Santos *et al.*, 2023)

Benzaldehyde	3.78	7.943	C_7H_6O; 240	PS, PP, PE, Nylon	(Lou *et al*., 2022, Santos *et al*., 2023)
WWTP (Sediment)					
Heneicosyl heptafluorobutyrate	72.78	41.09	C25H43F7O2; 13932483	Polytetrafluoroethylene (PTFE)	(Leslie *et al.*, 2022)
1,19-Eicosadiene	23.32	48.681	C20H38; 519006	PE	
AA (SW)					
Benzene, ethenyl-	25.15	6.004	C_8H_8; 7501	PS	(Santos *et al*., 2023)
2,4-Hexadiyne	24.78	2.282	C_6H_6; 137727	ABS plastic, cellulose acetate plastics	(Santos et al., 2023)
Benzene, methyl-	6.54	3.453	C_7H_8; 1140	PS	(Santos *et al*., 2023)
Benzaldehyde	5.26	7.943	C_7H_6O; 240	PS, PP, PE and nylon	(Lou *et al*., 2022)
AA (Sediment)					
Hexacosane	59.27	32.724	C26H54; 12407	PE	
Octacosane	27.43	32.27	C28H58; 124008	PE	

Tetratetracontane	9.7	31.52	C44H90; 23494	PE	(Jaafar *et al.*, 2022)
			IA (SW)		
Tetratetracontane	9.94	29.56	$C_{44}H_{90}$; 23494	PE	(Jaafar *et al.*, 2022)
Hexacosane	9.22	31.554	$C_{26}H_{54}$; 12407	PE (HDPE/LDPE pyroltics)	(Jaafar *et al.*, 2022)
1-Hexanol, 2-ethyl-	5.09	9.916	$C_8H_{18}O$; 7720	PE, PVC	(Leslie *et al.*, 2022)
1-Heptadecene	4.5	31.786	$C_{17}H_{34}$; 23217	PE	(Leslie *et al.*, 2022)

3.4 Discussion

The presence of MPs in surface water has been well-documented (Selvam *et al.*, 2021, Hossain *et al.*, 2022, Shafi *et al.*, 2024). However, most of the studies have focused on their occurrence and role in transporting the pathogens within these environments. The impact of pollution sources on the type and abundance of MPs has not been well documented. The uMsunduzi River is an essential waterway in KwaZulu-Natal, South Africa which is attributed to various sources, each contributing to the deterioration of water quality. The pollution sources include industrial waste, agricultural runoff, urban activities, and wastewater discharge. This study exhibited a variety of physicochemical parameters that are indicative of the water quality. The pH values observed in this study (7.22 to 7.51) are within the South African Department of Water Affairs, the US Environmental Protection Agency range (USEPA), and the World Health Organization (WHO) (2017, 2021) (Affairs and Forestry, 1993, Holmes, 1995) (Table 3.3) for diverse uses such as irrigation, domestic purposes, and recreational purposes.

The pH levels, below 6.5 and above 8.5, can be influenced by industrial effluents, wastewater discharges, and urban runoff, leading to fluctuations that may harm aquatic life (Rahman *et al.*, 2021). Previous research on the uMsunduzi River by Gemmell and Schmidt (2013) and Magwaza (2017) revealed pH ranges of 7.18 to 8.47 and 7.14 to 8.7, respectively, which were consistent with the values found in this study.

The DO is a critical parameter for the health of aquatic ecosystems, serving as an indicator of water quality and the river's ability to support aerobic life forms (El Morhit and Mouhir, 2014). In this study DO concentrations were critically low for the AA and UA-impacted sites (2.3 mg/L and 2.8 mg/L respectively) as shown in Table 6 as an indication of poor water quality. A previous study reported in the uMsunduzi River demonstrated 4.38 to 5.26 mg/L concentrations (Magwaza, 2017), which were slightly higher levels than the DO concentration found in this study. The requirement

for DO concentration ranges from 4 to 6 mg/L (Water and Organization, 2004, Organization, 2006, Edition, 2011, Organization, 2017), concentrations of 3-4 mg/L or lower have been associated with unhealthy water environments, which can be influenced by a variety of factors, including temperature, organic pollution, and the presence of nutrients (El Morhit and Mouhir, 2014). Therefore, the low DO level in this study was ascribed to a high level of contamination from different pollution sources.

The SC and TDS are crucial parameters that reflect the ionic composition and salinity of water. This study indicated moderate levels of dissolved ions (Table 3.3), indicating pollution impact. Furthermore, the COD levels ranged from 89.67 to 108.33 mg/L, reflecting the presence of significant amounts of organic and inorganic pollutants. These pollutants consume oxygen during decomposition, contributing to the observed DO levels. According to the South African Water Quality Guidelines, COD levels in natural river systems should ideally be below 30 mg/L to minimize ecological impact (Affairs and Forestry, 1993, Holmes, 1995, Holmes, 1996). The elevated COD values in this study far exceed this standard, highlighting the severe pollution in the water bodies.

3.4.1 Type and abundance of MPs associated with pollution sources

Microplastic pollution was ubiquitous along the uMsunduzi River, as evidenced by a total of 208 particles/L which were recovered in the surface water from all the sites. However, there was high abundance of MPs in the IA-impacted site (69 particles/L) compared to other sites (Figure 3.2). This site is impacted by industrial activities, hence previous studies have shown that one of the factors contributing to the prevalence of MPs in the aquatic environments is the industrialization along the coastal shelf of KwaZulu-Natal (Ryan *et al*., 2018). Furthermore, the high plastic debris in South Africa's aquatic environments is found mostly near industrial areas (Julius *et al*., 2023).

Furthermore, the second highest was observed in the WWTP-impacted site (51 particles/L), which is impacted by wastewater discharge, therefore wastewater discharges can be considered as the second significant source of MP pollution in this river. The impact of wastewater discharges on MP contamination of rivers has been studied and reported extensively (Murphy *et al*., 2016, Conley *et al*., 2019, Yu *et al*., 2020). Carr *et al* (2016), and Cheung and Fok (2017) reported that many small and light MPs can escape from WWTPs into the surface water through incomplete removal and a large volume of effluent which may not be captured by the treatment process (Wu *et al*., 2019a, Xu *et al*., 2020). It is therefore not surprising that this source has been identified in the current study to be a contributory source for MPs occurrence in the uMsunduzi River. Furthermore, several agricultural activities have been reported to result in the contamination of surface water bodies with MPs (Verma and Saksena, 2010, Kapp and Yeatman, 2018, Lang *et al*., 2022). This could be explained by the presence of MP in the AA-impacted site, which is impacted by agricultural activities.

Additionally, this study demonstrated the presence of MPs in sediment samples. There was a high abundance of MPs in sediment samples compared to MPs found in surface water. Looking at previous studies, it has demonstrated some rivers showed a high number of microplastics in sediment samples compared with water column (Ding *et al*., 2019, Jiang *et al*., 2019). This could be due to the accumulation of the MPs in the sediment due to their hydrophobic nature and reduced buoyancy because of microbial association resulting in an increase in density (Artham *et al*., 2009, Mammo *et al*., 2020). Additionally, these MPs could have settled in the sediments due to interaction with other solids in the river (Mammo *et al*., 2020).

3.4.2 Impact of pollution sources on the type and shape of microplastics

Fibers were numerically the dominant type among the categories of MPs collected at all sampling sites, as illustrated in (Figure 3.3A). Previous studies in freshwaters done by Uurasjärvi *et al.* (2020) and Uogintė *et al (*2022) showed similar results. Microplastic fiber types have been shown to be small threads and come from synthetic fabrics (Uurasjärvi *et al*., 2020, Uogintė *et al.*, 2022).

Therefore, wastewater discharge and industrial washing have been linked to the entry of microplastic fibers into rivers; thus, the direct discharge of untreated wastewater into the river from these metropolitan areas may be the main element at play (Browne *et al.*, 2011, Zhang *et al.*, 2017, Di and Wang, 2018, Yuan *et al*., 2019). Pellets were the second highest type of MPs, which is a primary MPs mainly used as raw materials for personal care products and manufactured plastic products, and were isolated from both surface water and sediment samples, indicating contribution from wastewater discharge and industrial effluent (Ding *et al*., 2019). Fragment-type particles are considered secondary and are created by the disintegration of larger pieces thrown away by tourists and local inhabitants (Uogintė *et al.*, 2022), as validated by field observations, as plastic debris floated about the uMsunduzi River. Furthermore, the IA-impacted site demonstrated the presence of foam in the samples. This foam originates from thermocol (expanded polystyrene) boxes, commonly used for fish storage and transportation in industrial and commercial settings (Malla-Pradhan *et al.*, 2022). The presence of foam in surface water is likely due to the air injected into foam, especially Styrofoam, making it less dense and allowing it to float. A study conducted by Osorio *et al* (2021) showed a high abundance of foam in the rivers Meycauayan and Tullahan in both surface water and sediment samples (Osorio *et al*., 2021).

3.4.4 Chemical characterization of MPs using FTIR and Pyro-GC/MS

The comparison of FTIR and Pyro-GC/MS results across all sites reveals consistent identification of key polymers, shedding light on the pollution sources and the pathway through which MPs enter the environment (Table 3.4). The FTIR spectra was used to identify MPs from different sites, where the chemical structures of different types of MP polymers differ, and their stretching and bending are impacted by the presence of carbon, hydrogen, and oxygen atoms (Thakur *et al*., 2023). Whereas the Pyro-GC/MS used distinct chemical profiles which were observed in the analysis of pyrolyzate compounds across various sites providing valuable insights into the types of polymers present and their probable sources, which are consistent with the expected pollution sources for each site. At the UA-impacted site, both FTIR and pyro-GC/MS analysis identified PE and nylon as major contributors to the MP load. The prevalence of nylon, a synthetic polymer commonly used in textiles, fishing nets, and engineering components used by urban communities daily, highlights the significant input from consumer activities in urban area. These findings align with previous studies that have found that urban runoff is a primary source of MP pollution in aquatic environments (He *et al*., 2022a). the detection of these polymers in both analytical methods underscores the robustness of these findings, confirming the persistence and widespread distribution of these materials in urban water bodies.

In the WWTP-impacted site, both methods identified a diverse array of polymers, including PE, PP, PS, and nylon. The presence of these polymers is an indication of diverse sources contributing to plastic waste entering the WWTP, including household and industrial waste which are major contributors to the influent of WWTPs (Ngo *et al.*, 2019). The PP is frequently used in packaging, automotive components, and consumer goods, while PS is widely used in disposable containers, packaging, and insulation materials (Lee and Bee, 2024). Nylon, predominantly used in textiles and ropes, suggests significant input from synthetic fabrics and related products (Horton *et al*., 2017,

Rajan *et al*., 2023). The FTIR results suggest that while some polymers may partially degrade or be retained during the treatment process, many are still discharged into the environment. This is corroborated by the Pyro-GC/MS findings, which detected similar polymers. The presence of these polymers indicates that WWTPs are critical points for the entry of diverse plastic wastes into the environment, highlighting the challenge of effectively removing MPs during the treatment process.

In the AA-impacted site, both FTIR and Pyro-GC/MS analyses revealed the presence of PE, PP, PS, and nylon, with additional detection of PVC by Pyro-GC/MS. These results suggest that agricultural practices contribute significantly to MP pollution. Modern agricultural practices involve plastics in a variety of ways, including mulches and polytunnels, which can degrade in situ, in addition to bale twine and wrapping, which can be improperly disposed of. These items can degrade to form secondary MPs within the environment (Horton and Dixon, 2018, Gherghel *et al*., 2019). Additionally, some practices involve the application of sewage sludge to agricultural land as fertilizer (Edo *et al*., 2020).

The IA-impacted site showed the presence of PE and PP as major polymers in both FTIR and Pyro-GC/MS analysis. The consistency between the two methods highlights the extensive use of these polymers in industrial applications, such as packaging, insulation, and manufacturing processes. The presence of PE is commonly used for packaging materials such as plastic bags, shrink wrap, and stretch films, essential for protecting and transporting goods (Horton *et al*., 2017). It is also employed in the production of pipes and fittings for chemical, gas, and water transport due to its chemical resistance and durability (Wang *et al*., 2020b, Wang *et al*., 2020a). Additionally, PE is utilized in manufacturing large containers and storage tanks for industrial liquids, as well as protective liners in equipment to prevent corrosion (Wang *et al*., 2020b). Its application extends to insulation materials, industrial films, and components like gears and bushings.

3.5 Conclusion

In this study, the magnitude of water quality in the uMsunduzi River was studied to evaluate the extent of water pollution in the river. It was found that the water pollution was mainly caused by anthropogenic sources. The water quality parameters showed contamination in the river, with the DO concentration critically low for the AA (2.3 mg/L) and UA (2.8 mg/L) and high values of COD ranging from 89.67 to 108.20 mg/L, indicating potential pollution from these sites. These findings underscore the need for addressing pollution sources in these areas. This emphasizes the intricate connection between land use and water quality.

The detection of fibers, fragments, and pellets provides additional evidence supporting the pervasiveness of MPs. Interestingly, fibers were the most dominant shape across all sampling sites for both surface water (60%) and sediment (59%) samples, which is particularly significant as it shows the association between pollution sources and MP types. Notably, higher concentrations of MPs at the IA-impacted site and the WWTP-impacted site further highlight that industrial and municipal wastewater streams are major contributors to MP contamination. The consistency of MP types in aquatic environments (surface water and sediment) highlights the widespread and enduring presence of these contaminants which indicates that pollution sources not only introduce MPs but also influence where they are found.

The combined use of FTIR and Pyro-GC/MS has provided a comprehensive understanding of the types and sources of MPs across different sites. Both methods consistently identified key polymers such as PE, PET, PP, PS, PC, nylon, and PVA, corresponding to specific sources of pollution and land use activities. At the WWTP-impacted site, the main polymers released are PE, PP, PS, and nylon. Similarly, the IA-impacted site showed a predominance of PE, PS, and

PP. In UA-impacted sites, PE and nylon were most prevalent, linking directly to urban community activities and textile-derived waste. For AA-impacted sites, PE, PP, PC, and PS

were detected. As for the sediment samples, both AA and WWTP showed the presence of PC in the FTIR, while the Pyro-GC/MS demonstrated PE for both samples. These findings emphasize the role of land use and pollution sources in determining the types of polymers that dominate MP pollution highlighting human activities and environmental contamination.

CHAPTER IV

4.0 ANALYSES OF MICROBIAL POPULATION DIVERSITY ASSOCIATED WITH MICROPLASTIC BIOFILM USING SHOTGUN METAGENOMIC SEQUENCING

4.1 Introduction

Microplastics are ubiquitous pollutants in aquatic environments, playing a significant role in altering microbial community structures through biofilm formation (Wang *et al.*, 2021b). These tiny plastic particles, less than 5 mm in size, provide a stable surface for microbial colonization, creating what is often referred to as the "Plastisphere." The biofilms formed on MPs offer a unique habitat that fosters microbial diversity, including both benign and pathogenic species, with varying functional capacities (Yang *et al*., 2020c, Bhagwat *et al*., 2021). Understanding the factors shaping microbial community structure, and composition within aquatic ecosystems and the plastisphere is crucial.

Recent studies have explored the structural and functional composition of microbial communities in surface water and on MP surfaces (Li *et al*., 2021b, Wright *et al*., 2021, Zhao *et al*., 2021a), the knowledge of these microbial communities remains limited, particularly in the ecosystems influenced by various pollution sources. Recent findings suggest that the plastisphere promotes microbial turnover, disrupts ecosystem sustainability, and alters the microbial community composition in aquatic environments (Li *et al*., 2021b). Other studies have shown that abiotic factors, such as the type of plastic, as well as ecological factors, can modify both the plastisphere and surrounding microbiomes (Lear *et al*., 2021, Rüthi *et al*.,
2023, Marsay *et al.*, 2023). Additionally, anthropogenic activities and varying pollution

gradients not only increase MP abundance in aquatic ecosystems but also affect the composition and function of bacterial communities (Wang *et al*., 2020c). This study aims to investigate the microbial community composition and diversity between the surrounding surface water and MP biofilms within the uMsunduzi River influenced by different pollution sources. The findings of the present study will explore the impact of different pollution sources on microbial communities, offering insight into how different environments contribute to microbial diversity in both surrounding surface water and the plastisphere.

4.2 Materials and Methods

4.2.1 Microbial community structure and function in surface water and plastisphere

4.2.1.1 Sample collection and processing

Microplastics from surface water were collected from the uMsunduzi River during the summer (February-March) and winter (July-August) seasons. To assess the microbial community in the surrounding surface water, 200 ml of surface water was collected and transported on ice to the laboratory for analysis. For the study of microbes associated with MP surfaces, ten liters of surface water samples were filtered through different mesh sieves (as mentioned in Chapter 3). The MPs retained on the sieves were transported to the laboratory on ice for further microbial characterization.

4.2.1.2 Microplastics isolation and DNA extraction

The filtered residues from the mesh sieves were rinsed using phosphate buffer saline (PBS) solution (pH 7.4) and further washed off into a glass beaker. The PBS with residues was filtered through 1.2 µm pore-size glass fiber-filtered papers using a vacuum system. Each filter paper was divided into four quadrates, to easily count the number of MPs on filter paper under the light microscope with a magnification of 4x (Nikon, Y-TV55 microscope). The filter papers were cut into smaller pieces to transfer into beat beads from the PowerSoil DNA extraction kit. The beat beads were placed on the rotor bead for 10 min at medium speed to rupture all the cells attached to MP surfaces. Thereafter, DNA was extracted from MP surfaces following the manufacturer's manual. Quantification and purity testing of DNA was done using the Nano spectrophotometer (Implen, USA).

4.2.1.3 Metagenomic sequencing

DNA fragmentation of the extracted DNA was performed by Covaris M220 and the obtained fragments (400bp) size were used to construct a paired-end library using NEXTFLEX Rapid DNA-Seq as per manufacturer's guidelines. An Illumina NovaSeq 6000 (Illumina Inc., San Diego, CA, USA), was selected to sequence the paired-end library via with NovaSeq Reagent Kits. For the obtained raw metagenomic data, we used Trim Galore (Chen *et al.*, 2019b) to trim the adapter sequences ($l < 50$ bp and N bases) and to remove low-quality ($q < 20$) reads. SqueezeMeta was used to perform the taxonomic analysis, functional identification and quantification (Tamames and Puente-Sánchez, 2019). The MEGAHIT (Li *et al.*, 2015) was used, to assemble the sequences and selected the contigs ($l \geq 300$ bp) for further taxonomic, functional annotation and gene prediction. MetaGene (Noguchi *et al.*, 2006, El Allali and Rose, 2013), was employed to perform ORFs (Open reading frames) prediction of the assembled contigs, and genes with length ($l \geq 100$bp) were retrieved and translated into amino acid sequences. We used CD-HIT (Chen *et al.*, 2016) and SOAP aligner (Li and Durbin, 2009, Hurgobin, 2016) to construct a non-redundant gene catalog and to calculate gene abundance based on 90 % and 95% similarity threshold, respectively, and the gene abundance was indicated using Reads Per Kilobase Million (RPKM). Finally, the taxonomic and functional gene annotations were obtained by aligning the representative sequences to NCBI, NR and KEGG databases (e-value: $1e^{-5}$), using diamond (Buchfink *et al.*, 2015).

4.2.1.4 Statistical analysis

All the statistical analysis of the obtained data was done using different packages in R v4.2.3 (R Core Team 2016). Observed, Simpson, Shannon and Chao1 indices were used to estimate species abundance and richness and alpha diversity. Wilcoxon and Kruskal-Wallis rank sum tests were used to test the significance of differences of community composition between the sampling sites and habitat. Bray-Curtis distance-based unconstrained principal coordinate

analysis (PCoA) was used to observe the differences in community composition and function between different sampling sites and habitats (surface water vs plastisphere) using a vegan package (Oksanen *et al.*, 2018). To evaluate microbial compositions across the sites and habitat, the PERMANOVA with 999 permutations using adonis function from the vegan package (Xia and Sun, 2023). In case where the homogeneity of variances was not fulfilled between the groups, ANOSIM was performed using Anosim function in vegan package (Oksanen *et al.*, 2018). Mantel and redundancy analysis (RDA) were performed to evaluate the effects of different environmental variables on microbial communities both across the sites and habitat. Spearman's correlation was used to estimate the relationship between the microbial diversity and the physiochemical properties corrplot function in R v 4.2.3. The machine learning based random-forest model (Wen *et al.*, 2021) was applied to obtain important biomarkers both across the sampling sites and between the plastisphere and surface water. The environmental difference and the corresponding community similarities between sites and surface water and plastisphere were use calculated using linear regression model. Using Spearman's rank correlation with coefficients greater than 0.6 ($\rho > 0.6$) and p-value < 0.05 used to construct the networks. The niche breadth values of species across the habitats and sampling locations were calculated via spaa package (Zhang *et al.*, 2022c). Sloan's Neutral based model was applied to investigate the community assemblage of surface water and plastisphere. A null model was applied to measure the significance of deterministic and stochastic processes in community assemblage using NST package.

4.3 Results

4.3.1 Bacterial diversity and composition

The present study investigated the microbial community composition and diversity between surrounding surface water and MP biofilms within a riverine ecosystem influenced by different pollution sources. The sampling adequacy was confirmed using rarefaction curves, indicating that the microbial populations were sufficiently captured across the different sites. Overall, the surrounding surface water contained significantly higher microbial richness compared to the biofilm on MPs (Wilcoxon, $p=0.0012$), with surrounding surface water hosting around 7% more bacterial taxa on average than MP surfaces (Figure 4.1a). This pattern was consistent across various sampling locations, such as UA, WWTP, AA, and IA impacted sites (Kruskal-Wallis, $p=0.00011$) (Figure 4.1b).

Bray–Curtis-based cluster analysis between the samples revealed that the bacterial communities in the surrounding surface water and plastisphere from different sites formed two separate clusters. Principal coordinated analysis (PCoA) revealed that the microbial community composition in the plastisphere and surrounding surface water differed significantly ($p < 0.05$) in the first and second dimensions for all sampling sites (Figure 4.2a) and habitat (surrounding surface water and MP surfaces) (Figure 4.2b). Permutational multivariate analysis of variance also showed significant differences in community composition both between the sites (PERMANOVA $F = 4.5709$; $R2 = 0.13$, $p < 0.001$) and habitat (PERMANOVA $F = 16.386$; $R2 = 0.15$, $p < 0.001$). Geographic proximity calculated in terms of beta diversity also exerted a significant effect on microbial communities with different samples clustering based on their sites, and habitat.

The dominant bacterial phyla detected included *Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes*, and *Cyanobacteria*. Notably, significant seasonal variations were observed, particularly in the relative abundances of *Proteobacteria, Acidobacteria, Fusobacteria, Bacteroidetes*, and *Actinobacteria* between winter and summer ($p < 0.05$), further influencing the microbial composition across both surface water and MP biofilm. These seasonal changes are likely associated with changes in environmental parameters such as temperature, nutrient levels, and hydrological conditions, which alter microbial colonization patterns and persistence impacted the microbial community composition across both surface water and MPs, with distinct profiles observed between habitats and sampling sites (Figure 4.3).

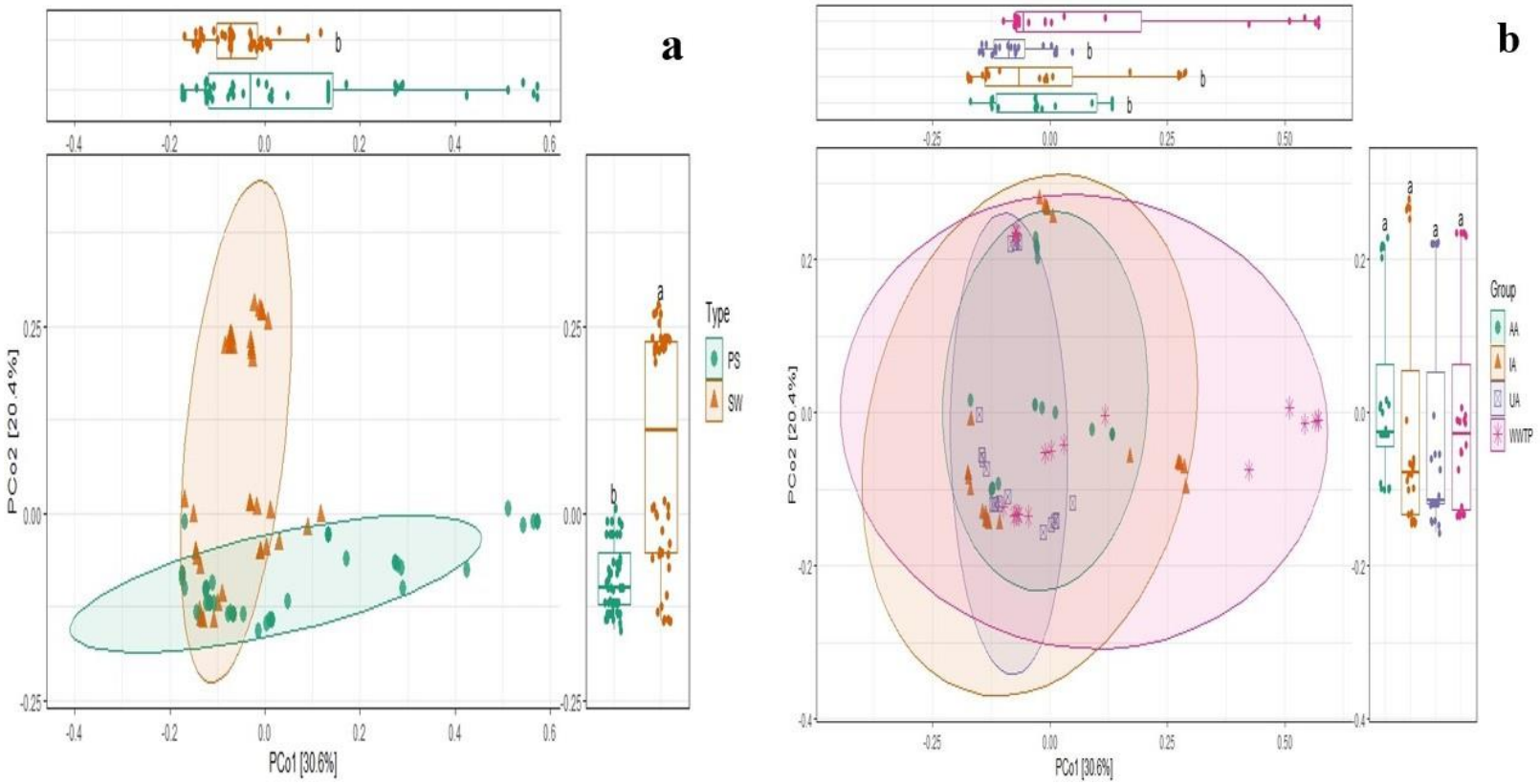


Figure 4. 1: Bacterial community: (a) habitats (surface water and MPs surfaces) and b) different pollution sources.

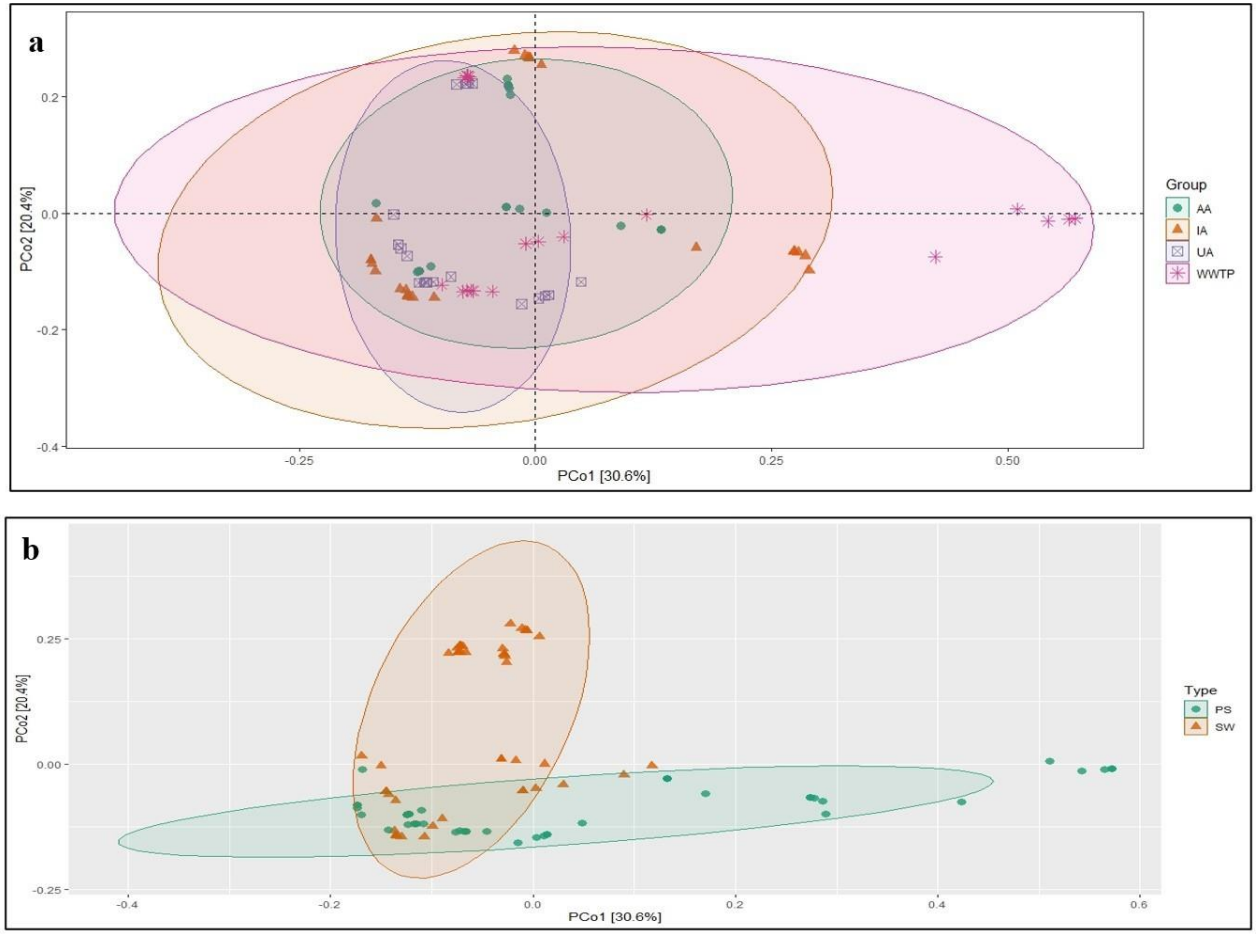


Figure 4. 2: PCOA analysis depicting differences in bacterial communities, (a) across sites, (b) habitats (surface-water and plastisphere)

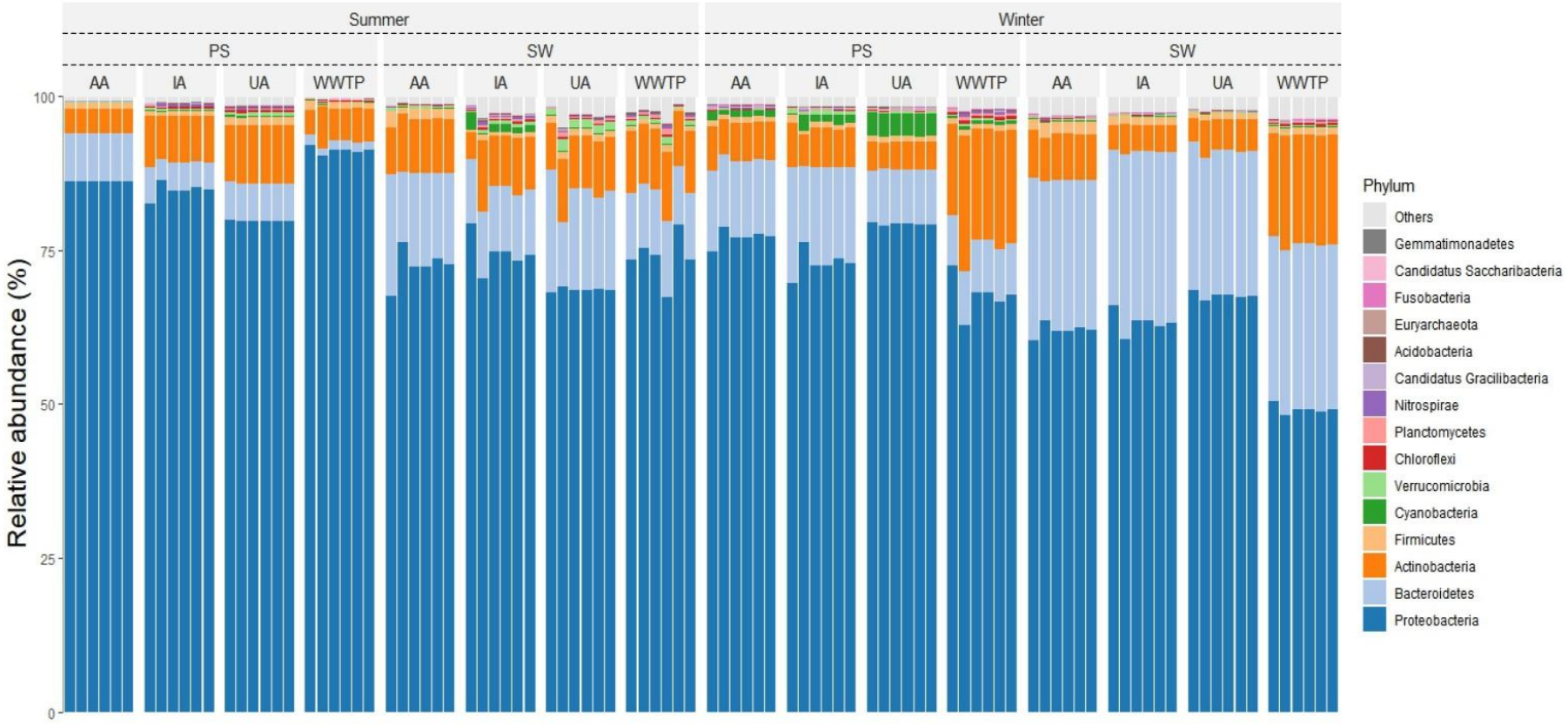


Figure 4. 3: Bar plot showing the relative abundance of bacterial communities across the sampling sites, habitats, and seasons.

4.3.2 The Unique, differentially enriched bacterial taxa and biomarkers of surface water and plastisphere

Differences in community structure were estimated between the surrounding surface water and plastisphere. LEfSe analysis (LDA >3) was used to identify the differentially abundant taxa that varied between the sampling sites, habitat, and season. First, the LEfSe analysis identified 20 discriminative bacterial taxa with significant ($p < 0.001$) variations in their relative abundance between the sites (IA, UA, WWTP, and AA impacted sites), habitat (surrounding surface water and plastisphere) and season (summer and winter). The samples from the WWTP-impacted site were differentially enriched with *Actinobacteria*, *Actionomycteia*, *Corynebacteriales, Micrococcales, Erwiniaceae,* and *Mycobacteriaceae* (Figure 4.4a). On the other hand, the samples from the IA-impacted site group, exhibited a significant presence of only 1 genus (*Undibacterium*), while samples from the AA-impacted site showed the significant presence of *Comamonadaceae*, *Moraxellaceae*, *Weeksellaceae*, *Moraxellales, Actinobacter* (Figure 4.4a). Similarly, the samples from the UA-impacted site exhibited the presence of *Betaproteobacteria*, *Oxalobacteraceae*, *Chromitiaceae*, *Sphaerotilus, Burkholderiales*, *Chromatiales*, *Sphaerotius*, and *Acidovorax* (Figure 4.4a). LEfSe analysis showed the presence of unique bacterial taxa in both habitats (surrounding surface water and plastisphere) and seasons (summer and winter), these taxa accounted for a considerable proportion (Figure 4.4b and 4.4c). In the case of habitat, surrounding surface water had more different taxa (52) than the plastisphere (21) and found a significant difference ($p < 0.05$) with surface water-enriched bacterial groups out-numbering the plastisphere-enriched taxa.

a

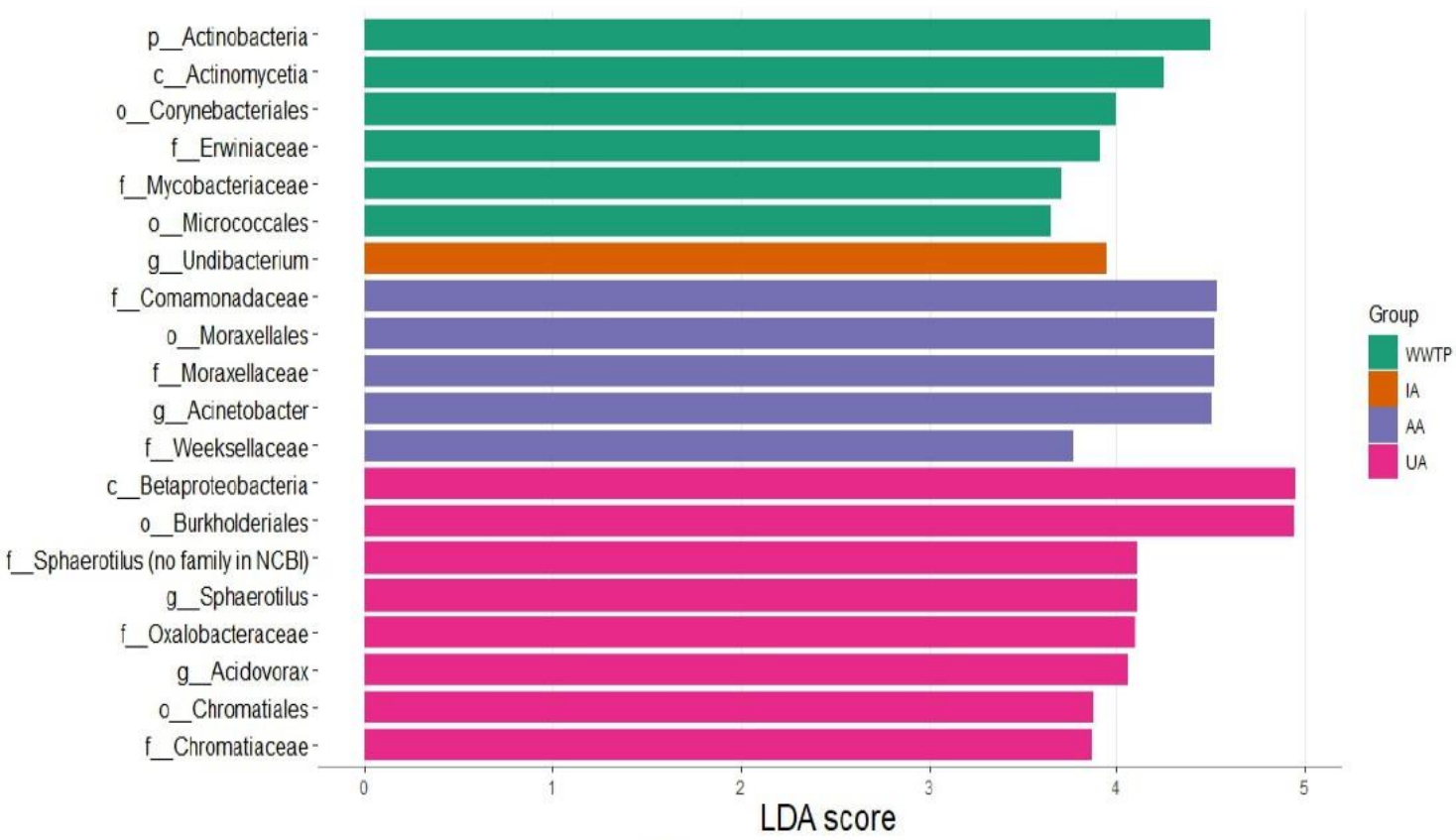

p__Actinobacteria
c__Actinomycetia
o__Corynebacteriales
f__Erwiniaceae
f__Mycobacteriaceae
o__Micrococcales
g__Undibacterium
f__Comamonadaceae
o__Moraxellales
f__Moraxellaceae
g__Acinetobacter
f__Weeksellaceae
c__Betaproteobacteria
o__Burkholderiales
f__Sphaerotilus (no family in NCBI)
g__Sphaerotilus
f__Oxalobacteraceae
g__Acidovorax
o__Chromatiales
f__Chromatiaceae
0
1
2
3
4
5
LDA score
Group
WWTP
IA
AA
UA

Fig.3.

b

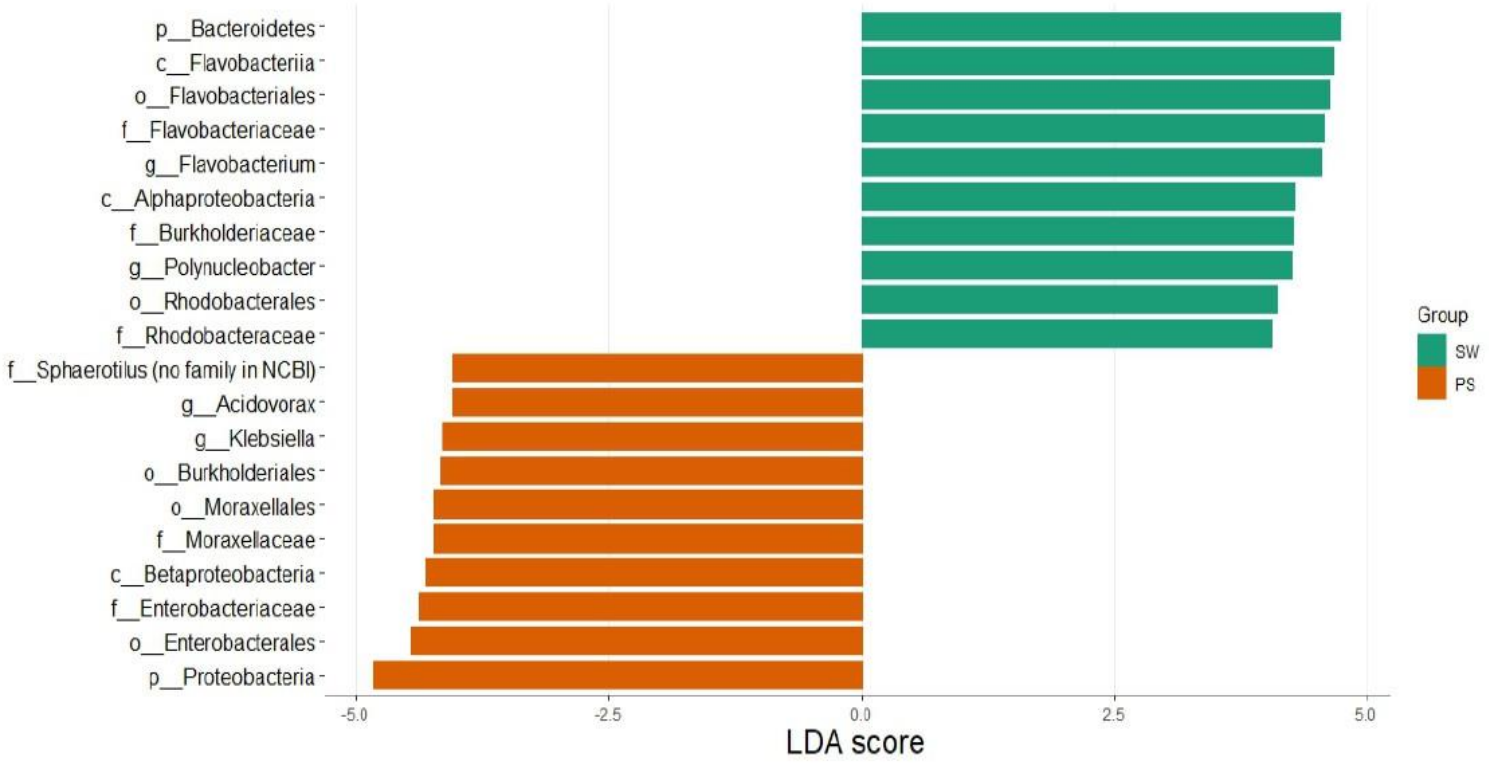

p__Bacteroidetes
c__Flavobacteriia
o__Flavobacteriales
f__Flavobacteriaceae
g__Flavobacterium
c__Alphaproteobacteria
f__Burkholderiaceae
g__Polynucleobacter
o__Rhodobacterales
f__Rhodobacteraceae
f__Sphaerotilus (no family in NCBI)
g__Acidovorax
g__Klebsiella
o__Burkholderiales
o__Moraxellales
f__Moraxellaceae
c__Betaproteobacteria
f__Enterobacteriaceae
o__Enterobacterales
p__Proteobacteria
-5.0
-2.5
0.0
2.5
5.0
LDA score
Group
SW
PS

Fig.3.

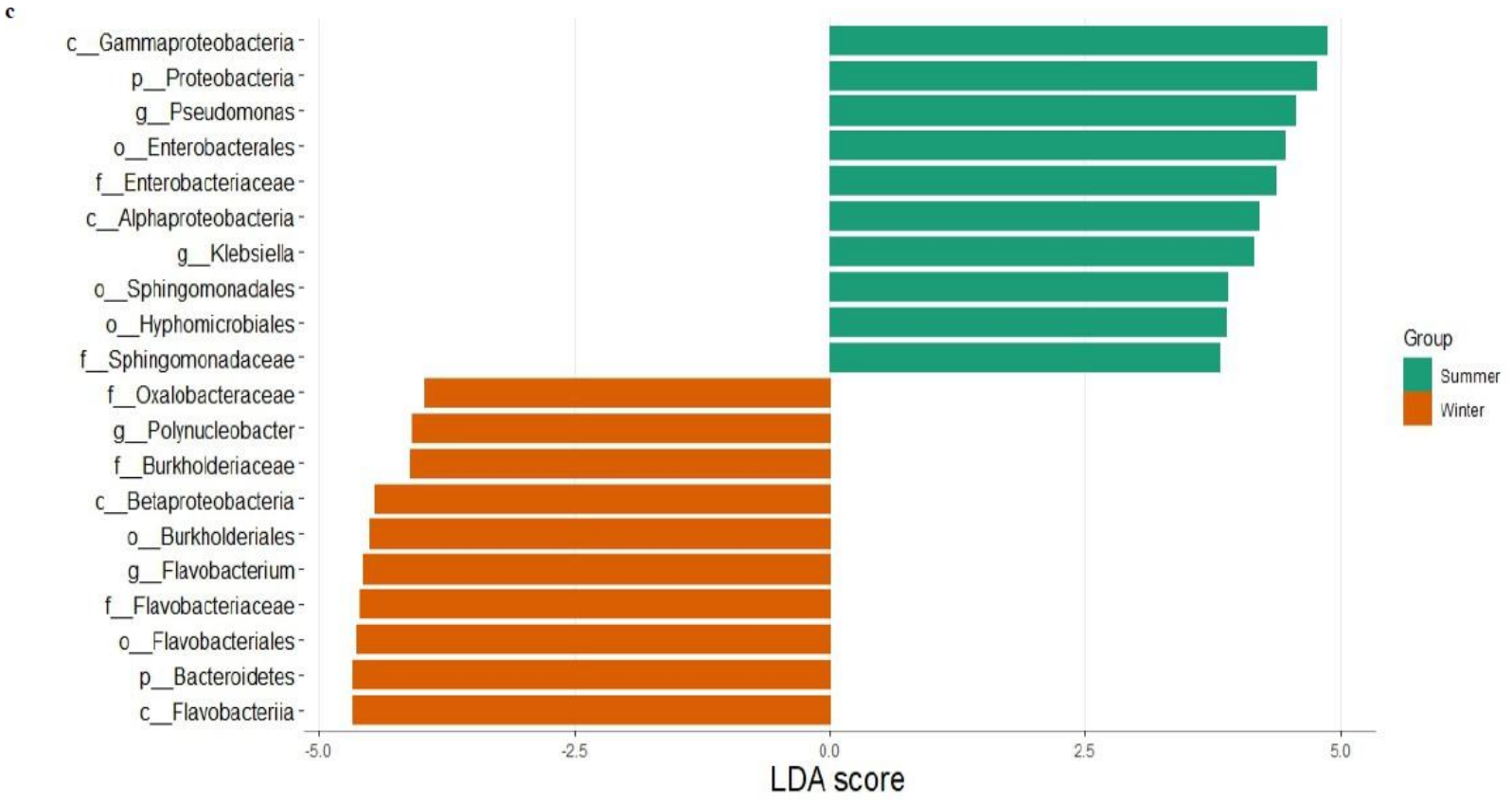


Figure 4. 4: Differential characteristics of microbiome between different: a) sampling sites; b) habitats (surface-water and plastisphere) and c) seasons (summer vs winter).

4.3.4 Correlation of environmental factors to microbial community

The study revealed that microbial communities in the plastisphere and surrounding environments are significantly positively associated with the type and source of pollution (UA-impacted site: R2 = 0.83, p = 0.015; WWTP-impacted site = R2 = 0.88, p = 0.0072, AA-impacted site: R2 = 0.85, p = 0.0075, IA-impacted site: R2 = 0.95, p = 0.0011) (Figure 4.5a-d). Mantel analysis showed that the bacterial communities in surrounding surface water were significantly correlated with i.e., DO (r = 0.62; p = 0.003) temperature (r = 0.50; p = 0.003), but were weakly related to salinity (r = 0.06; p = 0.26), pH (r = 0.019; p = 0.299), Specific Conductivity (r = 0.07; p = 0.192) and TDS (r = 0.032; p = 0.299). The plastisphere community was significantly correlated with DO (r = 0.33; p = 0.003), and salinity (r = 0.47; p = 0.003), but had a weak correlation with temperature (r = 0.07; p = 0.20), Specific Conductivity (r = 0.04; p = 0.38), and were negatively correlated with pH (r = - 0.045; p = 0.80) and TDA (r = -0.012; p = 0.49) (Figure 4.6a). These results suggest that DO and temperature are the key environmental variables driving the microbial community in surrounding surface water, on the other hand, DO and salinity are key environmental factors driving the microbial community in the plastisphere. Moreover, RDA showed that the microbial community both across the sites, plastisphere, and surrounding surface water was less constrained, as evidenced by environmental variables explaining 68.9% of the variations in the microbial community (Figure 4.6b, c). As is explained by the variations between two RDA axes; RDA1 explained 68.6 % and RDA2 explained 17.8% of the total variations. The water quality parameters explained a total of 86.4% variations in the microbial community composition both in sampling sites and habitats (surrounding surface water and plastisphere). Results showed that among parameters temperature (explains = 14.6 %, F = 12.96, p = 0.001), DO (explains = 5.3%, F = 4.68, p =

0.001), pH (explains = 3.1%, F = 2.54, p = 0.034), and salinity (explains = 7.2, F = 6.023, p = 0.002) were of significant impact, with the temperature being the most impactful parameter followed by DO. While the environmental variables such as TDS (explains = 1.9, F = 2.023, p = 0.338) and Specific Conductivity (explains = 1, F = 0.923, p = 0.766) were insignificant with lesser impact.

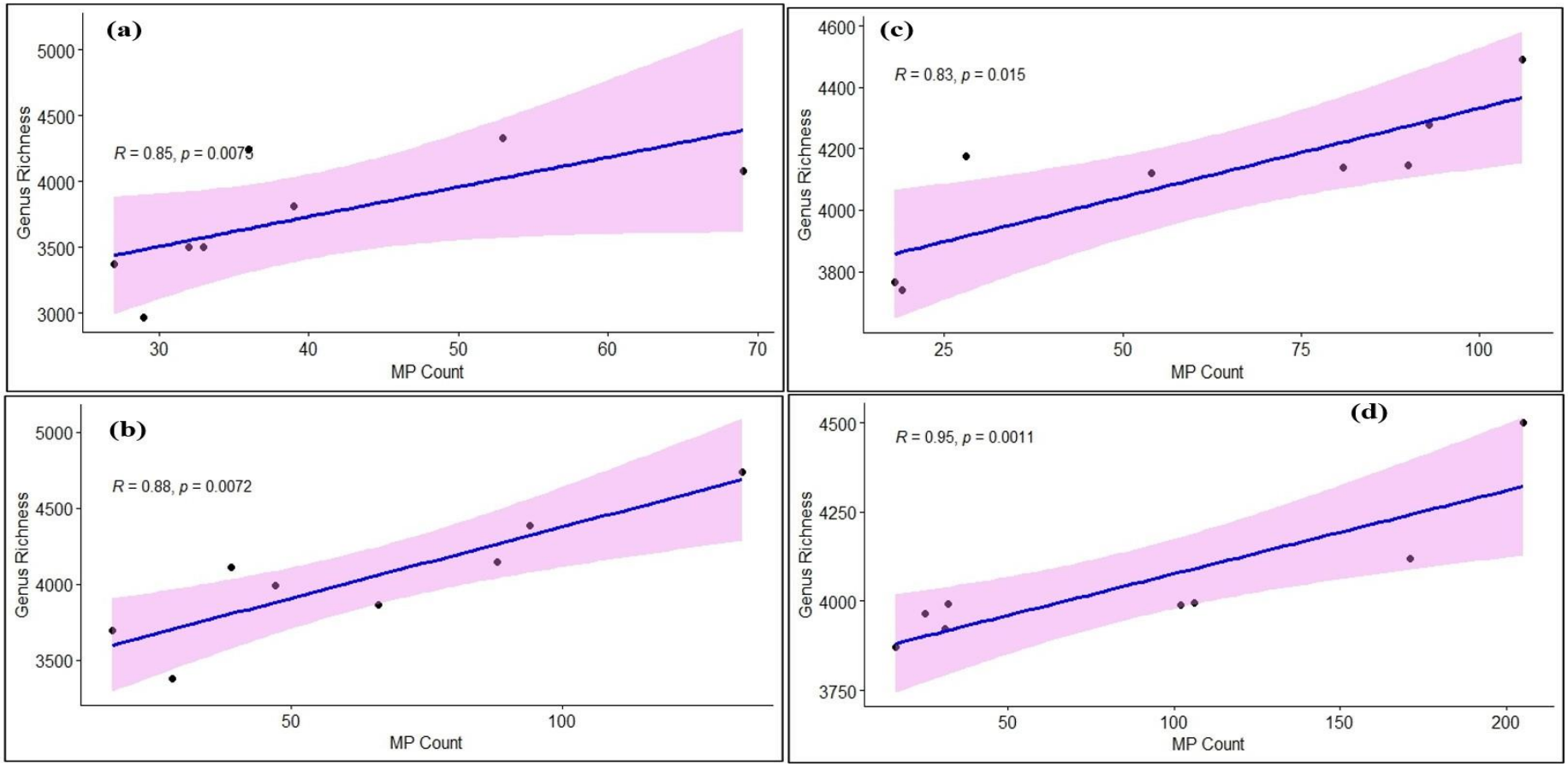
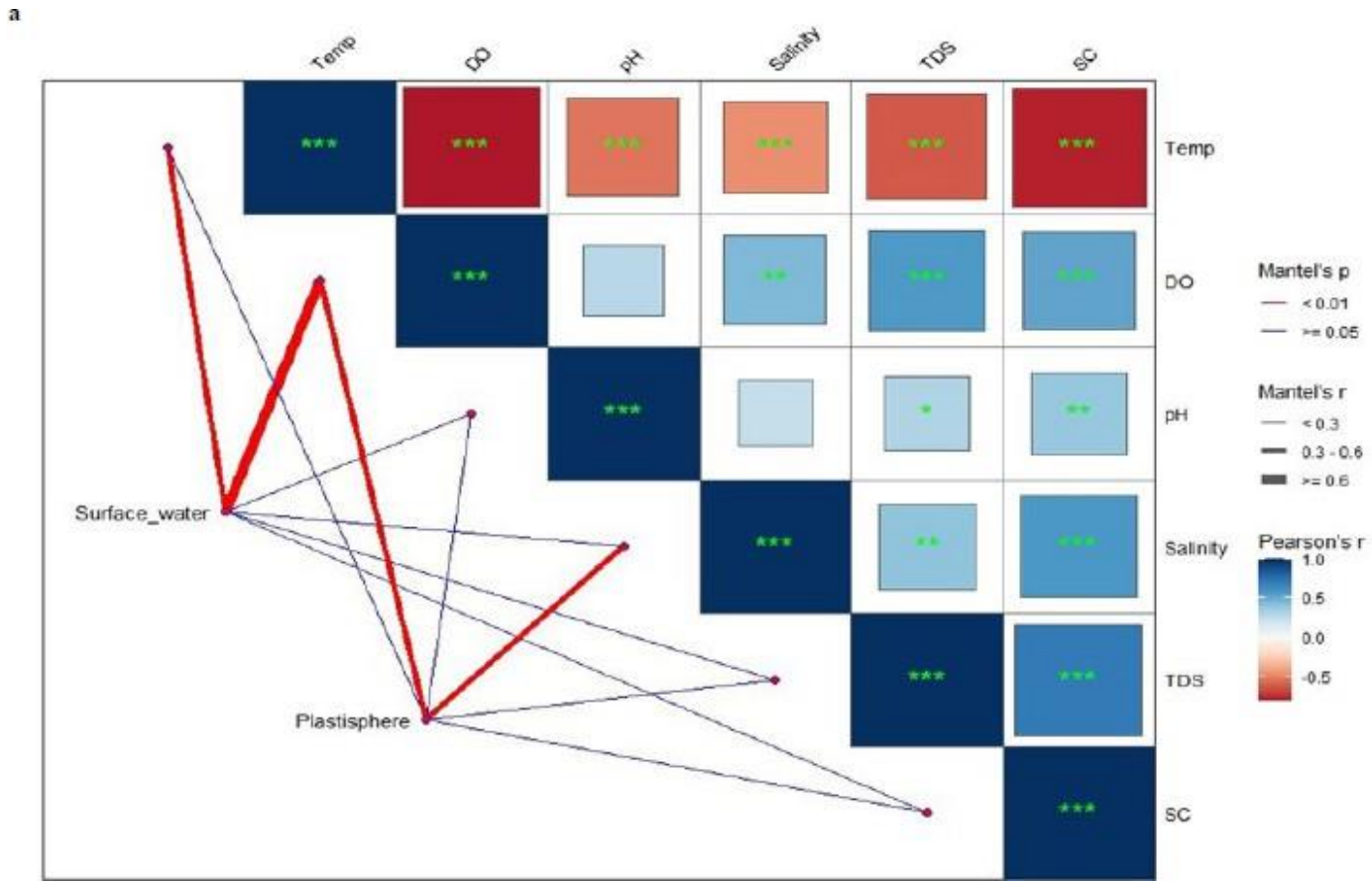
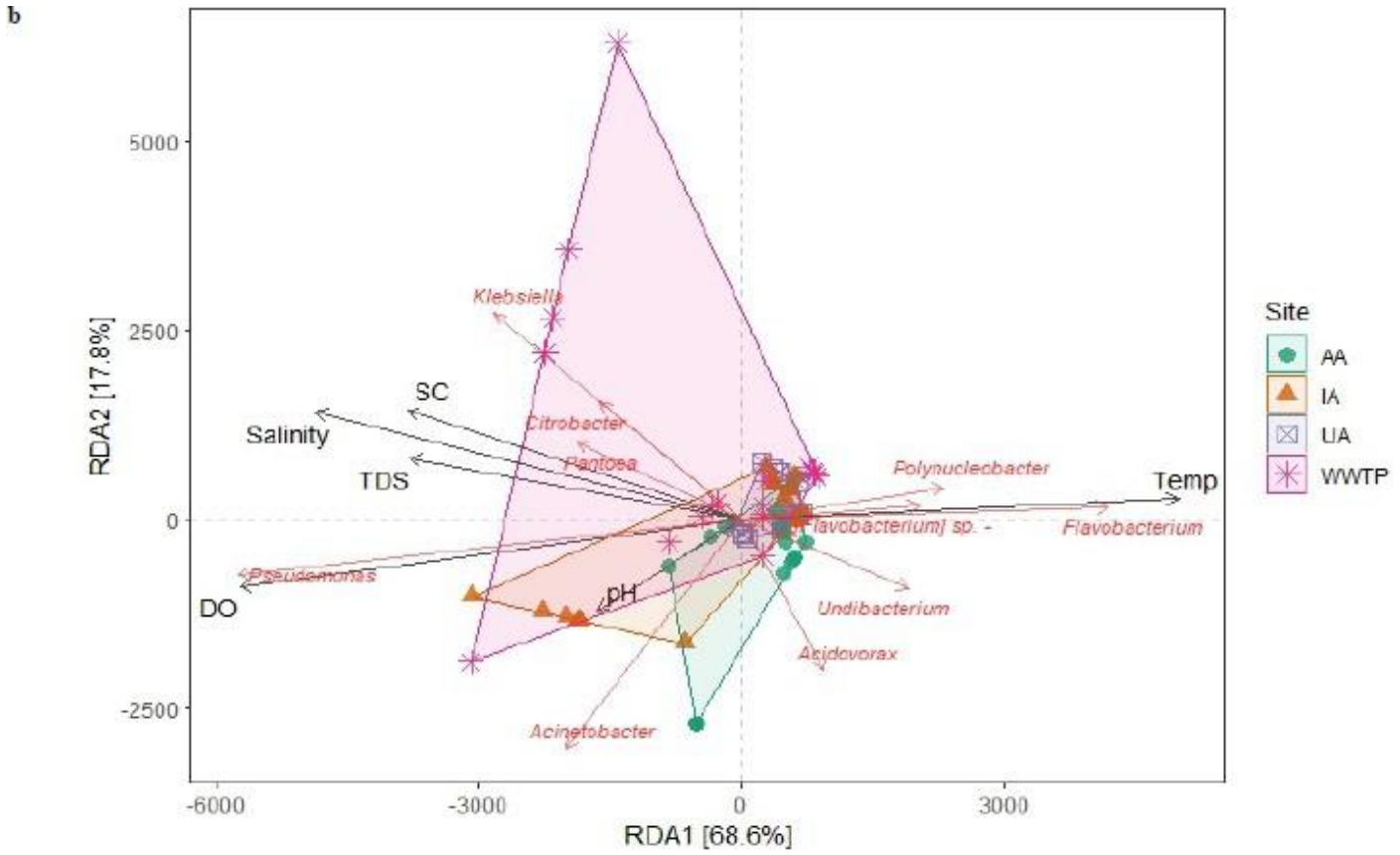


Figure 4. 5: Pearson correlation between microplastic (MP) and microbial diversity. Each dot represents a sample (a) UA site, (b) WWTP site, (c) AA site, (d) IA site.


a
Temp
DO
pH
Salinity
TDS
SC
Surface_water
Plastisphere
Mantel's p
< 0.01
>= 0.05
Mantel's r
< 0.3
0.3 - 0.6
>= 0.6
Pearson's r
1.0
0.5
0.0
-0.5


b
RDA2 [17.8%]
RDA1 [68.6%]
5000
2500
0
-2500
-6000
-3000
0
3000
Site
AA
IA
UA
WWTP
SC
Salinity
TDS
DO
pH
Temp
Klebsiella
Citrobacter
Pantoea
Polynucleobacter
Flavobacterium] sp. -
Flavobacterium
Pseudomonas
Undibacterium
Acidovorax
Acinetobacter

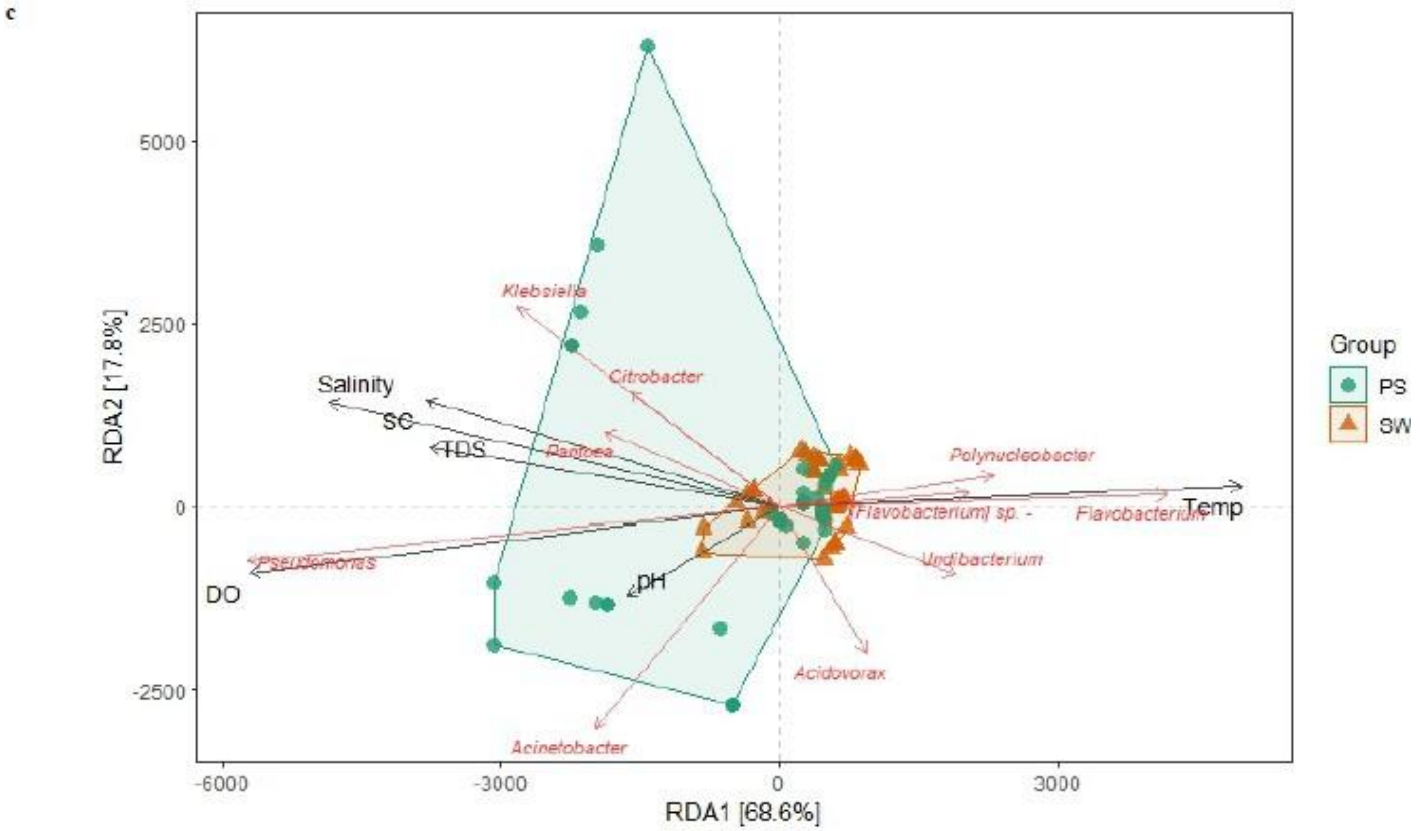


Figure 4. 6: Key drivers of variations in the surrounding surface water and plastisphere microbiome: (a) Mantel's correlation analysis between the physiochemical properties of water and surrounding surface water and plastisphere microbiome; (b) between different sampling sites; and (c) habitats; surface water and plastisphere.

4.4 Discussion

This study established that surrounding surface water and plastisphere harbored distinct microbiomes in a riverine ecosystem influenced by different pollution sources (Figure 4.1), which may affect the structural and functional dynamics of microbial communities within these environments. These findings are consistent with previous studies highlighting the niche differentiation of microbial assemblages on MPs compared to their surrounding environments (Zettler *et al*., 2013, Wang *et al*., 2020c, Yang *et al*., 2020c). One of the major reasons for this significantly diverse structure of microbial communities across the sampling sites may be attributed to the type and source of pollution (Sazykina *et al*., 2022). Based on these observations, the Bar plot revealed relatively abundant taxa that effectively distinguished among sampling locations and habitats, including surface water and plastisphere (Figure 4.3), supporting the idea that environmental pressures select for habitat-specific microbial assemblages. Furthermore, the seasonal dynamics are likely associated with changes in environmental parameters such as temperature, nutrient levels, and hydrological conditions, which alter microbial colonization patterns and persistence (He *et al*., 2022). Importantly, the ability of MPs to act as selective substrates may lead to the enrichment of specific microbial taxa, including potential pathogens or antibiotic-resistant bacteria, with ecological and public health implications (Kaur *et al.*, 2021). These findings emphasize the critical role of pollution source and seasonal variability in shaping MP-associated microbial communities in freshwater systems (Kaur *et al*., 2021, He *et al.*, 2022).

Recent studies have reported that microbial communities have prognostic values for determining the state and level of pollution as well as their biological safety (Philippot *et al*., 2024, Delogu *et al*., 2024). A second possible reason may be that MPs enter aquatic

ecosystems through the plastic cycle (i.e. through different environments) such as agricultural lands, wastewater treatment plants, industrial areas, urban and rural areas, municipal wastes etc, thus undoubtedly carrying microbes for long distances and through different environments (Wang *et al*., 2019, Birch *et al*., 2020). This in turn may drive changes in microbiome composition and function in the plastisphere and the surrounding water (Li *et al*., 2024). Likewise, the significantly distinct compositions of microbial communities in surrounding surface water and plastisphere may be because; first the plastisphere substrates are persistent, and mostly organic and hydrophobic in nature hence act as a filter for microorganisms (Li *et al*., 2021b, Zhang *et al*., 2022b, Miao *et al*., 2023). Second, plastics with large surface area readily adsorb various organic pollutants such as polyaromatic hydrocarbons (Sharma *et al*., 2020, Chen *et al*., 2023), pesticides (Wang *et al*., 2020d, Costigan

et al., 2022), metal derivatives, and polychlorinated biphenyls (Fu *et al*., 2021, Chen *et al*., 2023). All of these compounds are toxic and have the potential to affect microbial community composition (Wang e*t al.*, 2020b, Okoye *et al*., 2022). However, they can also serve as nutrients for other groups of microbes (Kooi *et al*., 2017, Li *et al*., 2021a). Consequently, MPs may act as a filter for such microbes, therefore, exerting selection pressure, resulting in a distinct microbiome in the plastisphere.

The study also established that the surrounding surface water microbiome was more affected by different environmental variables such as temperature, pH, dissolved oxygen, salinity, etc, than the plastisphere microbiome (Figure 4.6), These results suggest that microbial diversity and composition greatly depend on different environmental factors and the type of habitat (Yang et al., 2020c). The lesser influence of environmental factors on the plastisphere microbiome enables MPs to transport microorganisms and disseminate microorganisms from various pollution sources across different ecosystems. Based on the findings, it is reasonable to infer that the source of MPs and the particular environment they offer to microorganisms may more accurately account for the composition, diversity, and distinctiveness of the plastisphere microbiome. The noteworthy uniqueness and shift in the microbiome between surface water and plastisphere may be attributed to the specificity of these microorganisms to particular environments (Sun *et al.*, 2023). However, an essential aspect that warrants specific attention, is the potential invasion of ecosystems by unique microbial species carried by MPs from different sources (Amaral-Zettler *et al*., 2020, Bowley *et al*., 2021, Li *et al*., 2024).

4.4.1. Potential impact of surrounding surface water and plastisphere microbiome on ecosystem functioning

In this study, the distinct structure of microbial communities between the sampling sites and habitats contributed to significant functional disparity between the surrounding surface water and plastisphere microbiome, demonstrating that MP source, type, and pollution have a potential impact on ecosystem functioning. A significant difference in functions associated with biogeochemical cycles such as carbon, nitrogen, and sulfur cycling both between the sampling sites and between surrounding surface water and the plastisphere was observed. This suggests that MPs affect the functional aspects of biogeochemical cycling in ecosystems by forming unique biofilm communities. The findings are in line with previous studies that report significant differences in these genes and functional pathways between the plastisphere and surrounding surface water (Li *et al.*, 2020b, Seeley *et al.*, 2020, Li *et al*., 2024), which in turn alters the elemental biogeochemical cycling (Seeley *et al*., 2020). Secondly, the plastisphere harbors diverse chemicals, with concentrations (106 times) higher than the surrounding environment (Malla *et al.*, 2023a, Shi *et al*., 2023), a possible reason for the significant abundance of genes associated with compound degradation functions (Amaral-Zettler *et al*., 2020, Wright *et al*., 2020). Moreover, the significant differences in plastisphere and surrounding surface water microbiota in sampling locations, suggest that plastisphere from different sources have different ecological impacts - a possibility not explored yet.

Microplastic is known to change the structure, composition, and diversity of the microbial gene pool, as is observed with an increased abundance of genes related to metabolism, key cellular functions and defense mechanisms (Rüthi *et al.*, 2023), suggesting increased substrate transport and extracellular enzyme secretion (Vaksmaa *et al.*, 2021, Séneca *et al*., 2021). More

interestingly the increased abundance of cell motility and signal transduction genes in the plastisphere corresponds to the higher abundance of *Burkholderiales* and *Rhizobiales* which require flagellar genes to form biofilms (Rossi *et al*., 2018, Rüthi *et al.*, 2023). Moreover, the genes coding for enzymes such as hydrolases, dehydrogenases, peroxidases, lipases, and oxygenases were more enriched in plastisphere than in surface water, implying their role in the biodegradation of different types of polymers (Daly *et al.*, 2021, Priya *et al.*, 2022).

4.5 Conclusion

The study revealed insights into the differences in microbial community composition and diversity between surrounding surface water and plastisphere in a riverine ecosystem influenced by various pollution sources. Surrounding surface water exhibited higher bacterial richness compared to MP biofilm, with site-specific and seasonal variations significantly impacting microbial diversity. The major phyla dominated across different habitats and sites were *Proteobacteria* (30-70%), followed by *Bacteroides* (2-15%) and *Actinobacteria* (2-7%). Furthermore, the study showed clear habitat and site-specific enrichment of bacterial taxa between surrounding surface water and the plastisphere, with significant seasonal variability. Surrounding surface water (52) consistently hosted more different and abundant bacterial groups compared to the plastisphere (21).

Additionally, a strong association between pollution source and microbial richness, along with the influence of key variables like temperature, dissolved oxygen, and salinity demonstrated that both anthropogenic inputs and natural factors shape microbial communities in these habitats. These findings provide a comprehensive understanding of how pollution and environmental conditions interact to drive microbial dynamics, offering important insights for future efforts in monitoring and managing aquatic ecosystems impacted by microplastic pollution.

CHAPTER V

5.0 HEALTH RISKS IMPOSED BY POTENTIAL PATHOGENS AND ANTIBIOTIC-RESISTANT GENES ASSOCIATED WITH MICROPLASTICS IN SURFACE WATER AND PLASTISPHERE

5.1 Introduction

Since MPs are widely distributed in a variety of ecosystems, such as aquatic environments, streams, lakes, sediments, the atmosphere, soil, and inhabited species, they have generated a great deal of attention as rapidly developing anthropogenic pollutants (Li *et al*., 2022). When MPs accumulate in the river, they generate an enduring matrix and new biological niches for microorganisms and form biofilm on their surfaces (Wang *et al*., 2020c, Li *et al*., 2022). The technique by which infections adhere to MPs in the aquatic environment is intricate. As MPs reach an aquatic environment, they rapidly alter the surface morphology and chemistry. This can help with the adsorption of materials from the surrounding water, including nutrients and organic waste (Mammo *et al*., 2020, Wang *et al.*, 2021b). Recent environmental studies have raised growing concerns about the exposure of polymer MPs to aquatic habitats.

Despite ongoing debate on the health risks associated with the so-called Trojan horse effect of MPs, numerous studies have shown that dangerous bacteria, including *Vibrio spp, Escherichia coli* (*E. coli*), and *Acinetobacter baumanii*, colonize MPs in aquatic environments (Tavşanoğlu *et al.*, 2020, Zhong *et al*., 2023). Furthermore, it has been observed that viruses such as the *Coronavirus, Norovirus,* and *Poliovirus* can survive for several days on MP surfaces

(Deboosere *et al.*, 2012, Zhong *et al*., 2023). However, studies on the dangers of pathogen-attached MPs to people are still in their infancy and need more research (Zhong *et al.*, 2023). Furthermore, ARGs have received a lot of interest recently because of their capacity to endure in the environment long after the bacterial cells that carry them are dead (Reddy *et al.*, 2022). In many circumstances, ARGs are preserved in the microbial population even after the antibiotic selection pressure is eliminated (Yang *et al.*, 2020a). Studies have shown that the ARGs can be transferred across microorganisms of clinical and environmental/community origin by HGT (Yang *et al.*, 2020a, Yang *et al.*, 2022). This causes the microorganisms to acquire a tolerance for all types of medicines and all levels of defence, including last-resort antibiotics (Galafassi *et al.*, 2021). Due to frequent pollution by antimicrobial compounds introduced via a variety of factors, including health care institutions, animal husbandry, waste disposal facilities aquaculture, and wastewater treatment plants in urban areas, which represent one of the primary reservoirs and become critical sources of ARGs spread into the environment, the urban river ecosystem serves as an ideal setting for the development, prevalent occurrence, and spread of diverse ARGs (Galafassi *et al.*, 2021, Yang *et al.*, 2022). This study aims to assess the health risks posed by potential pathogens associated with MPs in surrounding surface water and the plastisphere by using a Quantitative Microbial Risk Assessment (QMRA) framework. The study focuses on the absolute abundance of *Pseudomonas aeruginosa* and *Salmonella enterica* from metagenomic data to estimate the probability of infection. The QMRA evaluates health risks for both adults and children who engage in recreational activities, such as swimming and paddling, in the polluted waters of the uMsunduzi River.

5.2 Materials and Methods

5.2.1 Potential Environmental Risk of MPs

5.2.1.1 Metagenomic Data Processing and Analysis

Poor quality sequences (q < 20) and adapter sequences were trimmed for all samples using TrimGalore v0.6.2 (https://github.com/FelixKrueger/TrimGalore) with reads longer than 50 bp retained. Sequence data from all samples were co-assembled using the SqueezeMeta v1.6.2 pipeline (Tamames and Puente-Sánchez, 2019) by using the default parameters. The pipeline provided taxonomic assignments for assembled contigs, metagenome-assembled genomes (MAGs), and annotation of open-reading frames (ORFs) with PFAM, KEGG, and COG terms.

The SqueezeMeta project was imported into R 4.2.3 using SQMtools (Puente-Sánchez *et al*., 2020)` whereafter DESeq2 was used to normalize read-counts for taxa at various taxonomic levels. Normalized read counts were exported and used for downstream statistical analysis. Samples were also individually assembled using MEGAHIT v1.2.9 (Li *et al*., 2015) with default settings. The individual assemblies were queried for ARGs using PathoFact v1.0 with default parameters (De Nies *et al*., 2021). The ARG risk scores in surface water and plastisphere were calculated by using MetaCompare with default parameters (Oh *et al*., 2018). For the statistical analysis (refer to chapter 4).

5.2.1.2 Source of data on microplastics associated with pathogens

Currently, there is no standardized approach to systematically assess the potential environmental risk of MP associated with pathogen exposure. To address this gap, QMRA focuses on two key pathogens, *Pseudomonas aeruginosa,* and *Salmonella enterica,* which are commonly found on MP surfaces. Notably, these pathogens are on the WHO bacterial priority

list in 2024. The concentration of the selected pathogens was taken from the metagenomic data sequencing, providing their absolute abundance on the MPs. This data was instrumental in calculating the potential risk exposure. The isolated MPs (abundance of MPs from the previous section) from the selected sites were considered in calculating the QMRA, ensuring the possible risk assessment was based on the environmental samples. The use of absolute abundance in calculating QMRA can either overestimate or underestimate the risk since no viable cell data were being used (such as culture cells). Absolute abundance provides the total count of pathogens, including both viable and non-viable cells. Without data on viable cells, it's difficult to accurately determine the actual risk of infection, as only viable pathogens can cause disease. This can lead to overestimation if non-viable cells are included in the count, suggesting a higher risk than what exists. Conversely, it can lead to underestimation if viable cell concentrations are lower than the total count indicates, potentially overlooking significant exposure risks.

5.2.1.3 Hazard Identification

In this study, pathogens associated with anthropogenic sources (such as effluent discharge from wastewater treatment plants, runoff from agricultural sectors, contaminants from industrial and urban areas, and natural sources such as the excreta from livestock and wildlife) and the absolute abundance of pathogens associated with MP surfaces were considered. Although the health concern raised by the effects of MPs remains controversial, an array of studies have demonstrated that pathogenic bacteria such as *Vibrio spp, Pseudomonas spp., Salmonella spp*., and *Acinetobacter baumanii* colonize MPs in aquatic environments. However, research on the danger of pathogen-attached MPs to humans is still in its early stages and needs more study. This study focuses on the river swimmers and canoers at the uMsunduzi River, considering

Pseudomonas aeruginosa, and *Salmonella enterica* as the potential pathogens for the health risk assessment.

5.2.1.4 Exposure assessment

Exposure is a quantitative measure of the amount of a pathogen that a host consumes, breathes, or comes into contact with. It is copies detected frequently used to determine the pathogen's source, such as water, and the actual exposure event, such as inadvertent ingestion. This study focused on the oral route of accidental ingestion by swimmers and canoers.

Concentration of Pathogens on MPs

The concentration of pathogens on MPs was determined using metagenomics data (absolute abundance):

$$C = \frac{N(Total)}{N(MP)} \quad (1)$$

Where, C is the concentration of pathogens on MPs (microbes per MP), N_{Total} is the total number of pathogen gene copies detected (microbes), and N_{MP} is the total number of MPs in the sample.

Ingestion rate

The ingestion rate for different exposure scenarios (swimmer and marathon paddlers) was determined using the equation below:

For swimmers:

$$IR(swimmer/canoeing) = MP(conc) \times V(ingestion) \quad (2)$$

where IR is the ingestion rate of MPs (number of MPs per liter of water), MP_{conc} is the concentration of MPs in the water (MPs per liter), $V_{ingestion}$ is the volume of water ingested during swimming (liters per hour).

According to the WHO, in 2021, the swimmer's accidental ingestion of river water ranged from 170 to 179 ml per hour for children, and 87 to 210 ml per hour for adults. Furthermore, there was a previous study done on risk assessment at the uMsunduzi River looking at the exposure during canoeing. The estimated accidental ingestion of surface water during the training: children (15-18 years) 7.7-11.6 ml, adults (>18 years) 11.6-15.4 ml as shown in (Table 5.1).

Table 5. 1: Estimates of volumes ingested per swimming and canoeing events.

	Range volume (L)	Median volume (L)	Reference
Ingested during swimming			

Children	0.170-0.179	0.175	(Organization, 2021)
Adults	0.089-0.210	0.149	(Organization, 2021)
Ingested during canoeing			
Children (15-18 years old)	0.0077-0.0116	0.010	(Ngubane *et al.*, 2022)
Adults (>18 years old)	0.0116-0.0154	0.014	(Ngubane *et al.*, 2022)

Ingestion dose calculation

The ingestion dose (D) was calculated using the following:

$$D = C \times V \times IR \times ET \quad (3)$$

Where, *D* is the dose of pathogens ingested (microbes), *C* is the concentration of pathogens on MPs (microbes per MP), *V* is the volume of water ingested per event (liters), *IR* is the ingestion rate of MPs (number of MPs per liter of water), and *ET* is the Exposure time (hours).

5.2.1.5 Dose-response assessment

In the health impact evaluation, a dose-response model was used to evaluate the health impact for the identified hazards. The dose-response model is a mathematical connection that relates

the chance of a reaction (such as an infection, disease, or death) to the dose of pathogen absorption by the receptor (i.e. swimmers) by several pathways (e.g. direct ingestion, inhalation, or touch). In this study, the *Pseudomonas aeruginosa* exponential dose response model was used, while for *S. enterica* the beta-poisson model was used as shown in *Table 5.2.*

Beta-Poisson dose-response model:

$$Pi(d) = 1 - (1 + (\frac{d}{N50})(2^{\frac{1}{\alpha}} - 1))^{-\alpha} \quad (4)$$

Where 'pi (d)' is the risk of infection, and 'd' the concentration of pathogen ingested in a known volume of surface water, N_{50} is the number of pathogens infecting 50% of the exposed population and α is the kinetic parameter (constant).

Exponential dose response model:

$$Pi(d) = 1 - e^{-kd} \quad (5)$$

This equation describes the probability of infection 'pi(d)' following an individual exposure event, where k defines the likelihood of the infective agent surviving the host's defence and subsequently initiating infection, while 'd' is the amount (dose) of pathogen that is ingested.

Table 5. 2: Pathogens Dose-Response model and parameter values

Pathogen	**Dose (µ) unit**	**Dose-response model**	**Dose-response parameter values**	**Reference**

Pseudomonas aeruginosa	Absolute abundance	Exponential	K=1.05×10^{-4}	QMRA-Wiki site
Salmonella enterica	Absolute abundance	Beta-Poisson	a =3.18×10^{-01} N_{50} =3.71×10^{04}	QMRA-Wiki site

5.2.1.6 Risk characterization

The hazard identification, exposure assessment, and dose-response assessment results were combined to determine the probability of infection with the selected pathogens (*P. aeruginosa*, and *S.enterica*) based on the different exposure scenarios. The initial risks were determined per hour of exposure using Eqs. 4 and 5, the risk of infection from multiple exposures to these pathogens was modeled using the formula:

$$P1(A) = 1 - (1 - Pi(d))^n \quad (6)$$

where 'Pi(d)' is the risk of infection from a single exposure to the pathogens, and 'n' is the risk per person per number of exposures (Amoah, 2018). In this study, exposure times considered were 1 hour.

$$Pi(total) = 1 - ((1 - P1(A)1) \times (1 - P1(A)2) \quad (7)$$

Where Pi(total) is the combined risk of infection from exposure to pathogens through ingestion of droplets containing MP associated with the pathogens and surface water with pathogens (*P.aeruginosa*, and *S.enterica*), P1 $(A)_1$ is the infection risk resulting from the ingestion of MPs associated with pathogens, P1 $(A)_2$ is the infection risk resulting from the ingestion of surface water.

5.3 RESULTS

5.3.1 Prevalence of pathogens on surrounding surface water and plastisphere

In this study, a total of 17 pathogens were identified across habitats (surrounding surface water and plastisphere) and sites (UA, WWTP, AA, and IA impacted sites) in different seasons (Figure 5.1). The pathogens identified include *Acinetobacter baumannii*, *Pseudomonas fluorescens, Pseudomonas aeruginosa, Pseudomonas putida, Aeromonas caviae, Stenotrophomonas maltophilia, Aeromonas veronii, Aeromonas hydrophila, Citrobacter freundii, Escherichia coli, Achromobacter xylosoxidans, Klebsiella pneumoniae, Enterobacter cloacae, Mycobacteriodes chelonae, Ralstonia pickettii, Agrobacterium tumefaciens,* and *Mycobacteroides abscessus*. The bacterial community analysis at the species level reveals that *A. baumannii* was the most dominant pathogen across all habitats, sites, and seasons, particularly in summer. In summer, *A. baumannii* accounted for a significant portion of the bacterial community in both the plastisphere and surrounding surface water samples. *P. fluorescens* (IA and AA impacted site) impacted site, and *Pseudomonas putida* (WWTP and UA impacted site) displayed a strong presence, particularly in surrounding surface water during the summer season. In contrast, *A. caviae* (AA and UA impacted site) and *P. aeruginosa* (in all sites) were more prominent in the winter season, particularly in the plastisphere, indicating seasonal shifts in bacterial communities associated with these environments. The data further showed that *E. coli* and *K. pneumoniae* had higher relative abundances during both seasons, whereby they are more associated with surrounding surface water during summer compared to MP surfaces, and then during winter season more associated with MP surfaces than SW.

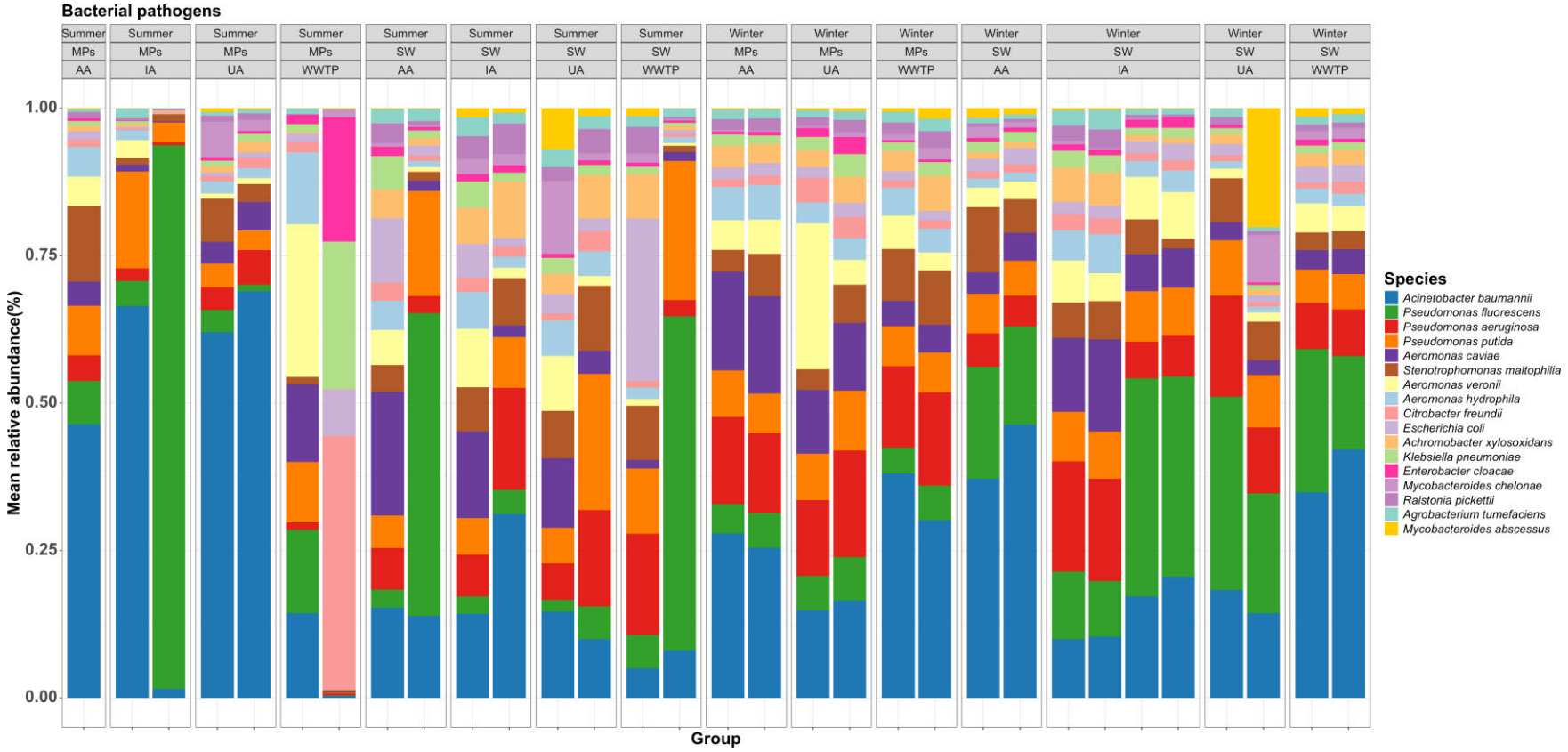


Figure 5. 1: Comparison in the relative abundance of top 17 pathogenic bacteria between habitat (surface water (SW) and plastisphere (PS)), different pollution sources (UA, WWTP, AA, and IA impacted sites) in different seasons.

5.3.2 Profiling of ARGs across sites UA, WWTP, AA, and IA in the uMsunduzi river

The ARGs were identified through metagenomic analysis, and the results were visualized using a heatmap. The heat map visually represents the presence of antibiotic resistance across various pollution sources, including UA, WWTP, AA, and IA impacted sites. In the heat map, each row represents ARGs, while each column corresponds to a different pollution source (Figure 5.2). The WWTP-impacted site exhibited the highest number of ARGs confer resistance, with a total of 11 major classes of antibiotic resistances including pleuromutilin, aminoglycoside, phenicol, nitroimidazole, fluoroquinolone, polymyxin, Fusidic-acid, MLS, glycopeptide, triclosan, and tetracycline. AA-impacted site followed this with 10 antibiotic resistance classes including rifamycin, diaminopyrimidine, bicyclomycin, Multidrug, Fosfomycin, beta-lactam, nucleoside, glycopeptide, sulfonamide, aminocoumarin, IA-impacted site with 6 ARGs included tetracycline, Antibacterial-free, peptide, elfamycin, sulfonamide, and aminoglycoside and UA-impacted site with 5 ARGs included acridine-dye, bacitracin, aminocoumarin, triclosan, and mupirocin.

Furthermore, the presence of ARGs varies significantly between surrounding surface water and plastisphere as shown in (Figure 5.3), reflecting their unique environmental conditions and contamination sources. In surrounding surface water, there were 9 dominant ARGs including categories such as diaminopyrimidine, aminoglycoside, mupirocin, and nitroimidazole, suggesting that surrounding surface water may have a distinct profile of antibiotic resistance, possibly influenced by different contamination sources and environmental factors. The plastisphere showed a broader array of 19 ARGs, including phenicol, fosfomycin,

pleuromutilin, polymyxin, elfamycin, bacitracin, MLS, fluoroquinolone, Multidrug, nucleoside, peptide, rifamycin, sulfonamide, tetracycline, antibacterial- free, bicyclomycin, fusidic-acid, and acridine-dye. This diversity indicates that the plastisphere serves as a unique habitat promoting the selection and maintenance of a wide range of ARG.

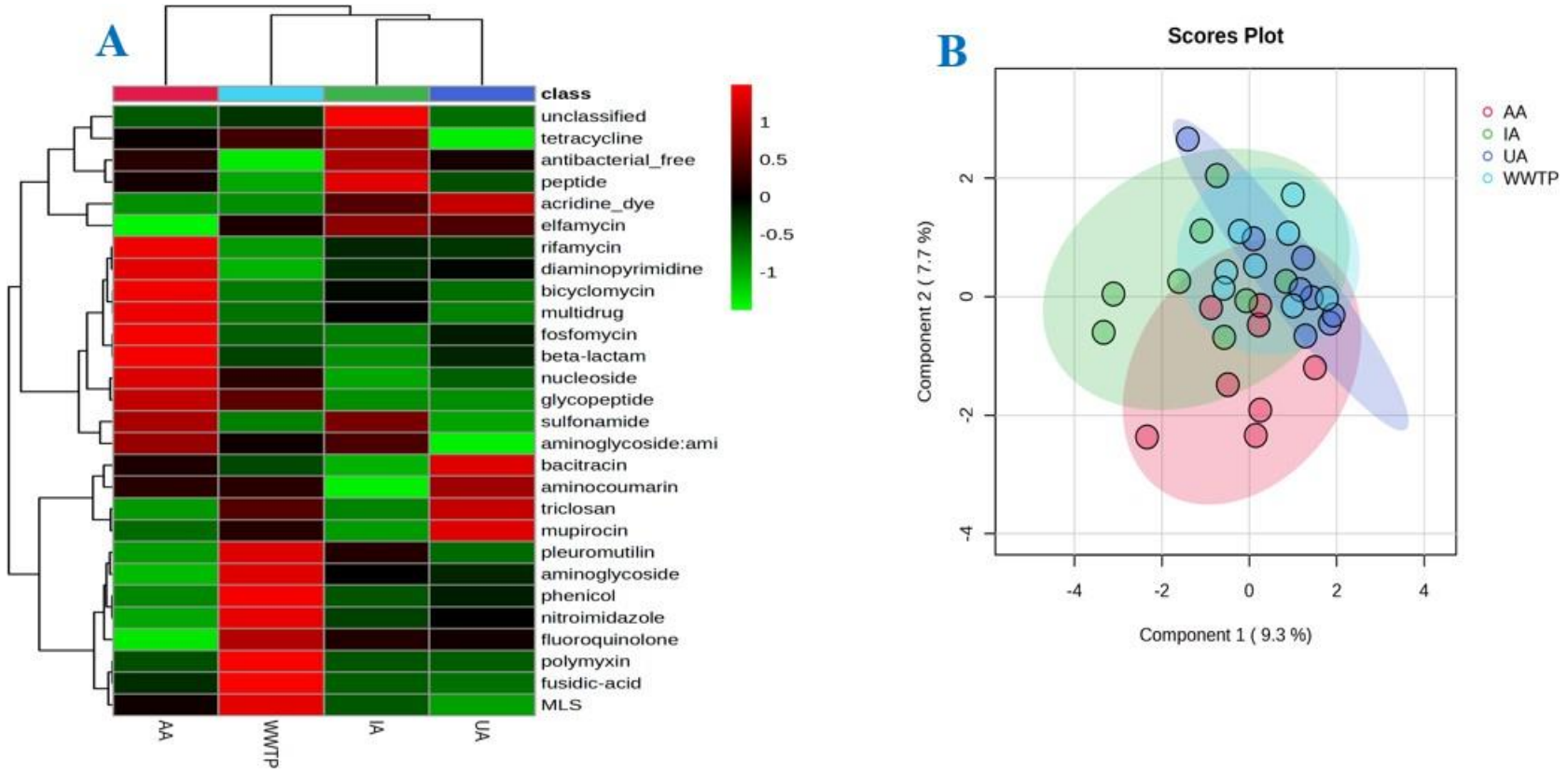


Figure 5. 2: Heat map showing the composition of antibiotic classes in the different sampling sites (A) and their principal component analysis (B).

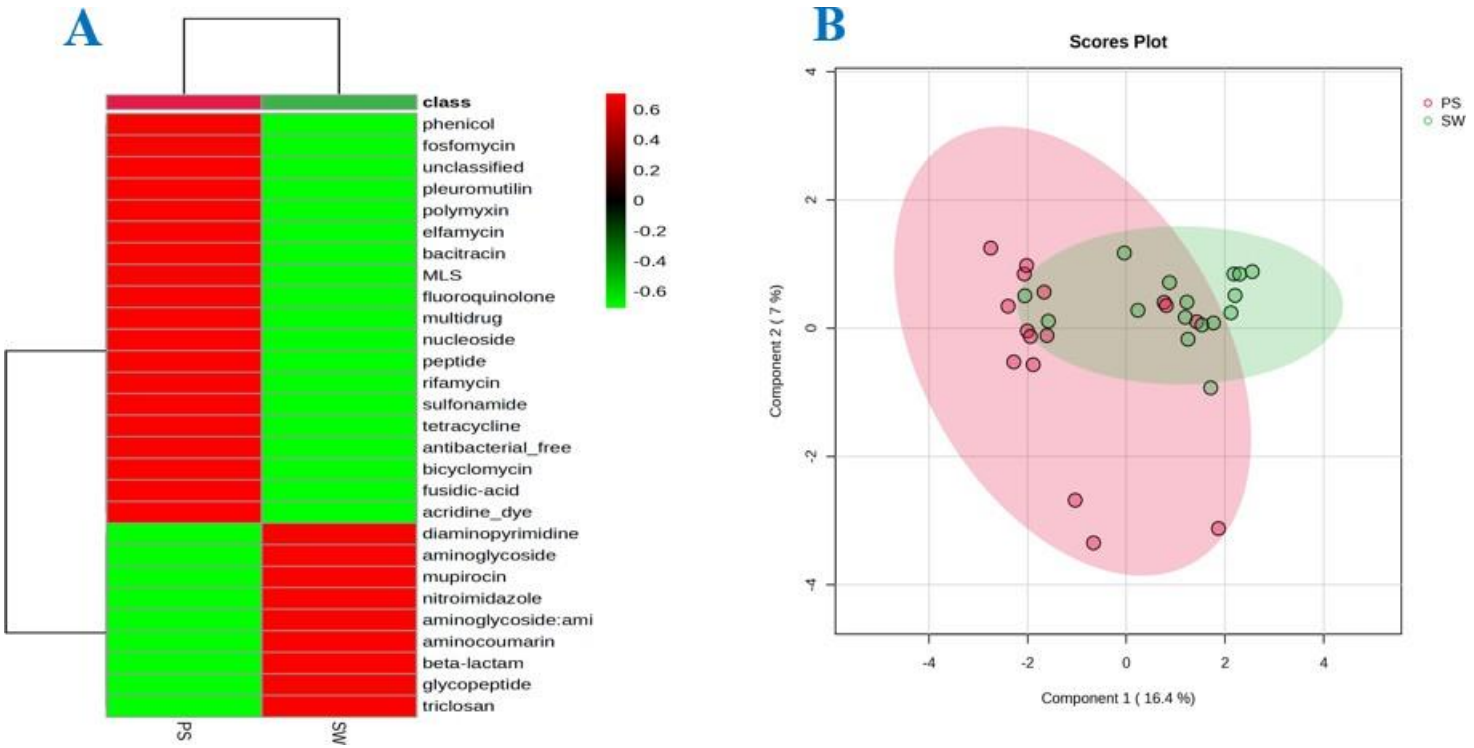


Figure 5. 3: Heat map showing the composition of antibiotic classes in the different habitats: surface water (SW) and plastisphere (PS) (A) and their principal component analysis (B).

5.3.3 Quantitative microbial risk assessment analyses

The probability of infection for *P. aeruginosa* and *S. enterica* was assessed across different sites (UA, WWTP, AA, and IA impacted sites) and seasons (Winter and Summer) for children and adults engaged in canoeing and swimming activities. The data show distinct seasonal and site-specific variations in infection probabilities.

Canoeing (Figure 5.4 A, B, C, and D): The probability of infection for children during winter varied across sites, with *P. aeruginosa* in surrounding surface water showing the highest infection risk at the WWTP-impacted site (0.3), while the lowest was observed at IA-impacted for *S. enterica* (0.05). In summer, the risk increased for most sites, particularly *P. aeruginosa* in surface water at the WWTP-impacted site (0.35), indicating a higher microbial load in warmer conditions. For adults, similar trends were observed, though the probabilities were generally lower than those for children, with a notable peak at the WWTP-impacted site during summer (0.4 for *P. aeruginosa* in surrounding surface water).

Swimming (Figure 5.5 A, B, C, and D): Swimming activities showed higher infection of probability compared to canoeing across all sites. In children, *S. enterica* in surrounding surface water during summer at the UA-impacted site recorded the highest infection probability (0.9), while *P. aeruginosa* in MP surfaces also shows infection risks across all sites. For, the combined risk was slightly lower but followed the same pattern, with the highest probabilities observed in summer.

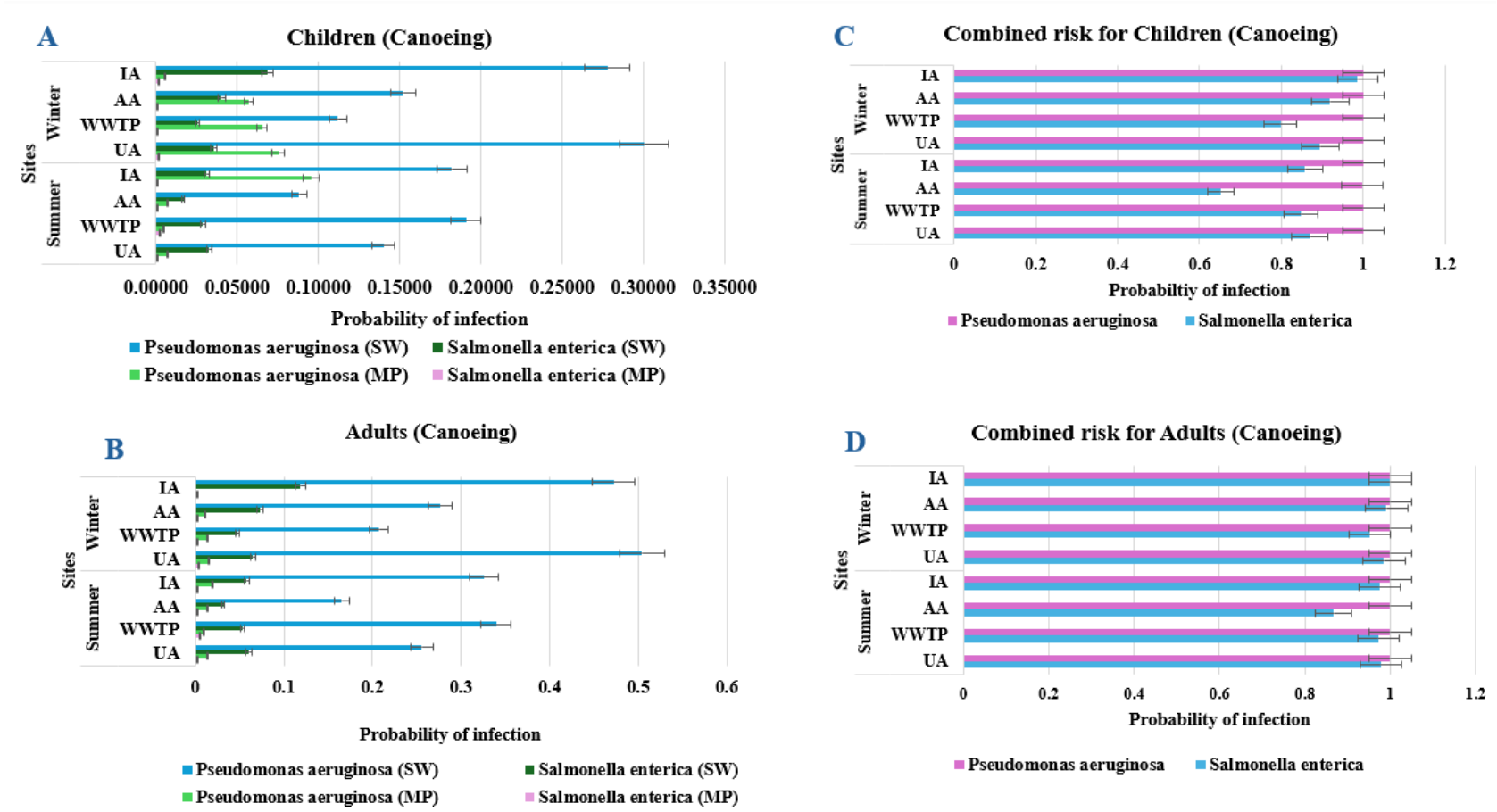


Figure 5. 4: Potential risks of infection with *Pseudomonas aeruginosa* and *Salmonella enterica* due to exposure while canoeing in the uMsunduzi River, analyzed across consuming A, B: SW or MPs (for both Children and Adults) and C, D: SW+MPs (for both Children and Adults) different pollution sites and seasons.

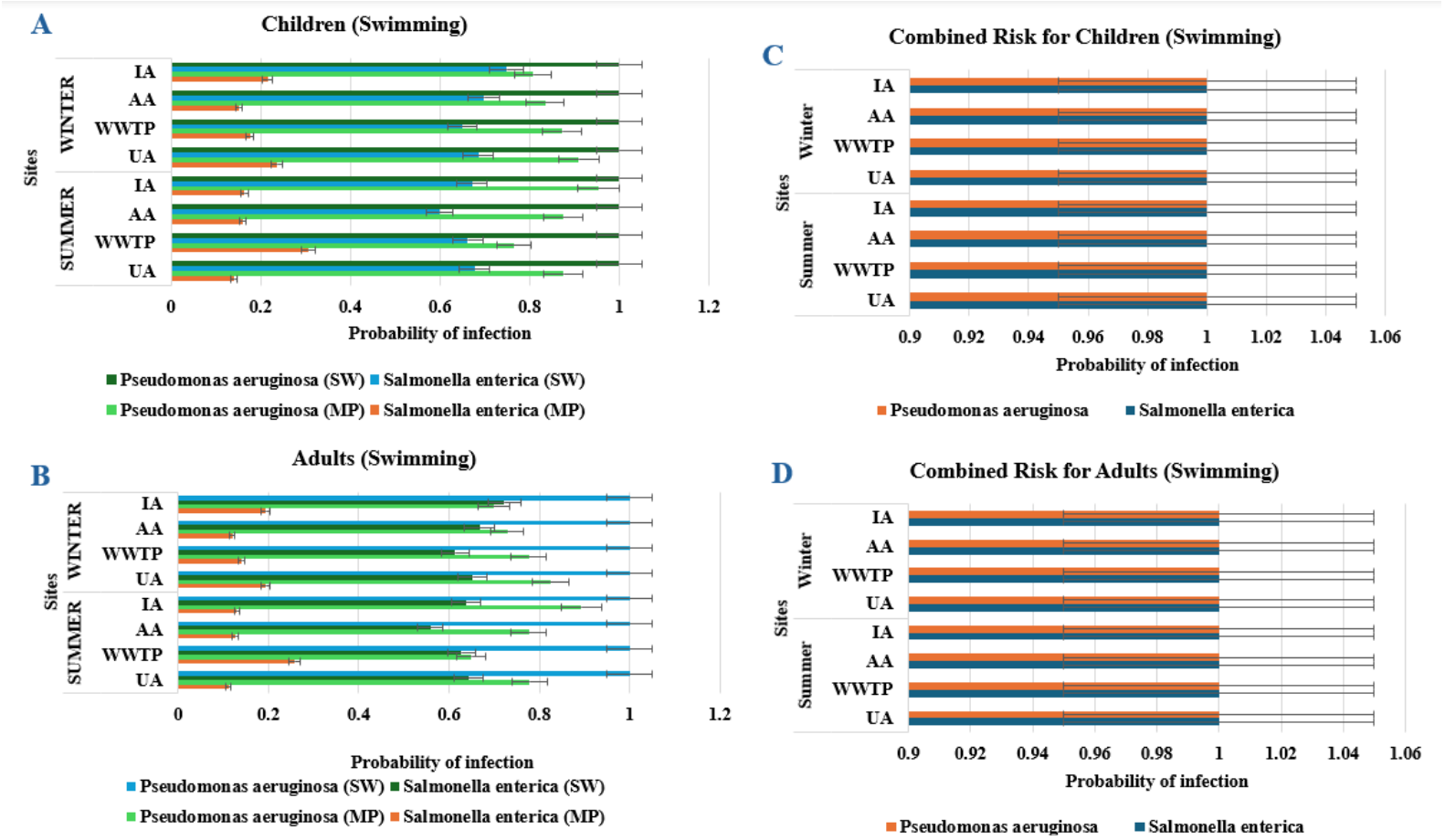


Figure 5. 5: Potential risks of infection with *Pseudomonas aeruginosa* and *Salmonella enterica* due to exposure while swimming in the uMsunduzi River, analyzed across consuming A, B: SW or MPs (for both Children and Adults) and C, D: SW+MPs (for both Children and Adults) different pollution sites and seasons.

5.4 Discussion

5.4.1 Distribution of pathogens across various pollution sources (UA, WWTP, AA, and IA), habitats (SW and plastisphere), and seasons

In this study, 17 major pathogens were identified across various pollution sources (UA, WWTP, AA, and IA impacted sites), habitats (surrounding surface water and plastisphere), and seasons highlighting the critical insights into their potential linkage with ARGs and the impact of environmental factors on their prevalence. The dominance of *A. baumannii* across all sites and seasons, particularly during summer, suggests that this pathogen may be a possible key carrier of ARGs. This bacterium is well-known for its multidrug resistance often harboring genes like *blaOXA*, which confer resistance to beta-lactam antibiotics (da Fonseca *et al.*, 2021, Gao *et al.*, 2023). The higher abundance of *A. baumannii* in the summer could be linked to warmer temperatures and increased organic matter, which are known to enhance the HGT of ARGs, especially in polluted environments like UA and WWTP impacted sites.

The presence of *P. fluorescens* in IA and AA impacted sites during summer, and *P. putida* in WWTP and UA sites, suggests that these species are known to possess resistance genes that confer tolerance to a variety of antibiotics, including fluoroquinolones and tetracyclines (Stapleton *et al.*, 2023, Wu *et al.*, 2024). These species are prevalent in environments with high levels of antibiotics (such as WWTP and AA impacted sites) and chemical pollutants (IA-impacted site), which may contribute to the selection and spread of ARGs. The presence of these microbes in the WWTP-impacted site further indicates their role in the persistence and dissemination of ARGs through treated effluent, which can enter natural bodies.

The prevalence of *A. caviae* in AA and UA impacted sites, along with the widespread *P. aeruginosa* across all sites during winter highlights the seasonal shifts in bacterial communities that may correlate with the distribution of ARGs. The *P. aeruginosa* is notorious for its

resistance to multiple antibiotics including aminoglycosides, beta-lactams, and quinolones, often due to the presence of genes including *mexB* efflux pump systems (Wang *et al.*, 2023b). Its ability to thrive in low-temperature conditions on the plastisphere suggests that it may act as reservoir for resistant strains during the winter, when other environmental pressures may reduce competition from less resilient microbes.

The observed seasonal variation in *E. coli* and *K. pneumoniae* between surrounding surface water and plastisphere also points to a dynamic interaction between environmental conditions and the persistence of ARGs. These microbes are among the members of the *Enterobacteriaceae* family which are common carriers of extended-spectrum beta-lactamases (ESBLs), which confer resistance to a broad range of antibiotics, including cephalosporins and monobactams (Goh *et al.*, 2023). Their association with MP surfaces during winter may be due to the protective niche these surfaces provide, enabling microbe's survival including the pathogens, and potential transfer in colder conditions when antibiotic degradation rates are slower.

Moreover, the presence of these pathogens in WWTP, AA, UA, and IA impacted sites highlight the role of these sites as critical points for the accumulation and dissemination of ARGs. The wastewater treatment plants are hotspots for ARGs due to the high concentration of diverse bacterial communities and sub-inhibitory concentrations of antibiotics, which promote the selection and horizontal transfer of resistance genes (Stapleton *et al.*, 2023). The release of treated effluents containing MPs and their associated resistant microbes into natural water bodies can further propagate ARGs across different environments, exacerbating the global issue of antibiotic resistance.

5.4.2 Distribution of antimicrobial-resistant genes across sites (UA, WWTP, AA, and IA sites) and habitats (surrounding surface water and plastisphere)

The significant difference in the number and variety of ARGs between surrounding surface water and the plastisphere suggests that the plastisphere acts as a more concentrated reservoir of antibiotic resistance. The detected ARGs confer resistance to 19 ARGs in the plastisphere compared to 9 in the SW (Figures 5.2 and Figure 5.3) indicating that MP surfaces possess a strong ability to absorb antibiotics, making the combination of these plastics and antibiotic contaminants a substantial threat (Stapleton *et al*., 2023). Additionally, MP surfaces provide a unique environment that facilitates colonization and also creates microhabitats where HGT can occur more readily, thus promoting the proliferation of ARGs (Jia *et al.*, 2024). The identification of ARGs such as multidrug, rifamycin, and sulfonamide on the plastisphere reflects the complexity of microbial communities associated with these synthetic surfaces, indicating HGT between the microbes.

A previous study done by Li *et al* (2018) examined the presence of five ARGs namely sulfadiazine, amoxicillin, tetracycline, ciprofloxacin, and trimethoprim on various MPs surfaces, including PE, PS, PP, nylon, and PVC (Li *et al*., 2018). The results showed polyamide-dominated ciprofloxacin, amoxicillin, and tetracycline. Additionally, the plastisphere, the microhabitat formed on MP surfaces, supports the survival of microbial communities. Microplastics also act as vectors, facilitating the transportation of ARGs (Rafa *et al*., 2023).In surrounding surface water, the ARGs detected are fewer but still significant, indicating that water bodies themselves are also reservoirs for antibiotic resistance, albeit to a lesser extent than the plastisphere (Sathicq *et al*., 2021). The types of ARGs found in surrounding surface water could be influenced by agricultural runoff, wastewater discharge, and other anthropogenic activities.

The presence of different ARGs across pollution sources highlights the varied impact of different pollution sources on the distribution of antibiotic resistance. The WWTP-impacted site with the most diversity of ARGs corresponds with prior studies that imply that wastewater treatment plants are hotspots for the accumulation of antibiotics from various sources as well as the growth of ARGs. This included antibiotics such as tetracycline, fluoroquinolone, aminoglycoside, and MLS which are the commonly found ARGs from wastewater treatment plant effluent that are persistent due to the presence influx of pharmaceuticals from domestic and industrial waste, creating an environment conducive to selecting and spreading resistant genes (Stapleton *et al.*, 2023).

In the AA-impacted site, the identification of ARGs such as Beta-lactam, Sulfonamide, and Rifamycin suggests a strong link to the use of antibiotics in livestock and crop production. The presence of tetracycline-resistant genes is particularly prevalent in agricultural soil and water bodies, as they are widely used as growth promoters and disease presentative in livestock (Yang *et al*., 2021). Sulfonamides are also common due to their extensive use in animal husbandry (Yang *et al*., 2021). Beta-lactams are regularly identified in agricultural contexts, particularly in dairy and meat production, where they are widely employed (Ferroni *et al*., 2020).

Overall, these results highlight the significant impact of seasonal, habitat, and site-specific factors on the distribution and abundance of bacterial pathogens. The association of these pathogens with MPs, particularly during winter, raises concerns about the potential for MPs to serve as reservoirs and vectors for harmful microbes from one point to the next, thereby posing risks to both environmental and public health. Further research is needed to fully understand

the mechanisms driving these associations and their implications in the context of environmental pollution and pathogen transmission.

5.4.3 Risk assessment

The data highlights several critical factors influencing the probability of infection in recreational water activities, particularly children and adults engaged in canoeing and swimming. The infection risk is significantly influenced by the type of water body (surrounding surface water vs. MP surfaces) and the season, with warmer conditions typically showing increased probabilities. This can be attributed to the higher microbial activity and replication rates during summer months, coupled with increased human activities that might contribute to the contamination levels (Zhang *et al*., 2021, Wang *et al*., 2021b). The *P. aeruginosa* in surface water consistently shows a higher risk than *S. enterica* across all sites, with the highest risk observed in the WWTP-impacted site as shown in Figures 5.3 and 5.4. This finding is consistent with its known ability to thrive in aquatic environments and form biofilms, enhancing its survival and pathogenicity (Mammo *et al*., 2020, Wang *et al.*, 2021b).

Furthermore, the elevated risk observed for the ingestion of pathogens from surface water and MPs during swimming scenarios for children compared to adults can be explained by their higher ingestion rates and longer exposure durations during water activities, which is consistent with findings reported in similar (Ngubane *et al.,* 2022). The role of MPs as a vector for pathogens is evident in the data, where *P. aeruginosa* showed significant infection risks on MP surfaces across various sites. These results support the previous studies that have shown that MPs serve as a habitat and transport medium for pathogens, increasing their persistence and potential for human exposure.

The contribution of combined risk assessment (MPs plus surrounding surface water exposure) to the overall infection of *P. aeruginosa* and *S. enterica* is more pronounced in both seasons, all sited, and for both adults and children. This highlights the importance of considering both surrounding surface water and MP surfaces in assessing microbial risks, especially in recreational water activities like canoeing and swimming. These findings underscore the need for improved water treatment and pollution control strategies to reduce the exposure risks posed by pathogens associated with MPs, particularly in urban and wastewater-impacted areas.

This study also highlights the critical public health threat posed by *P. aeruginosa* and *S. enterica*, particularly in the context of their association with MPs. According to the World Health Organization (WHO), both microbes are classified as one of the most critical AMR "priority pathogens", due to their remarkable ability to evade the effects of multiple antibiotics (Talebi Bezmin Abadi *et al*., 2019). These microbes' widespread presence in diverse environments, combined with its ability to thrive in various ecological niches, underscores their resilience and adaptability. The selective pressure exerted by sub-inhibitory concentrations of antibiotics in these environments has led to *P. aeruginosa* and *S. enterica* strains that are resistant to several major classes of antibiotics. This resistance not only complicates treatment strategies but also amplifies the risk of infection, particularly in environments where MPs may serve as reservoirs and transport mechanisms for these pathogens.

5.4.4 Limitations of using QMRA framework for assessing possible risk at uMsunduzi River through swimming and canoeing

While the probabilistic approach using the QMRA framework presented in this study provides valuable insights, several limitations must be acknowledged:

Data on Pathogen Concentration: The probability of infection was calculated using absolute abundance data derived from metagenomic analyses. This method inherently includes both viable and non-viable cells, which can lead to potential overestimations or underestimations in the calculated risk. Viable cells are the actual threat to human health, whereas non-viable cells do not pose a risk of infection, thus the inclusion of both in the absolute abundance data could skew the probability estimates. However, it is important to note that the primary objective of this study is not to deliver a precise estimation of infection risk but rather to establish a QMRA framework that can be applied to assess the potential health risks associated with pathogens linked to MPs in surface waters.

5.5 Conclusion

In total, 17 bacterial pathogens were identified across different habitats (surface water and plastisphere), sites (urban areas, wastewater treatment plants, agricultural areas, and industrial areas), and seasons (summer and winter). The MP surfaces (19) were found to harbor more ARG classes than the surrounding surface water, which contained only 9 classes, suggesting that MPs act as concentrated reservoirs for antibiotic resistance. The WWTP site exhibited a more diverse number of ARGs confer resistance, including tetracyclines, fluoroquinolones, and aminoglycosides, reflecting the influence of pharmaceutical effluents.

Finally, the QMRA evaluated the potential infection risks associated with *P. aeruginosa* and *S. enterica* due to exposure to surrounding surface water and biofilm-associated with MP surfaces in the uMsunduzi River. The results showed that the presence of *P. aeruginosa* and *S. enterica* on the MP surface significantly increases infection risks compared to exposure from surrounding surface water alone. MPs enhance pathogen persistence, distribution, and exposure, leading to elevated infection probabilities for both children and adults across all sites and seasons. These findings underscore the critical role of MPs as vectors, amplifying health risks in contaminated aquatic environments.

CHAPTER VI

GENERAL CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The study presented and supported the hypothesis that land use patterns/activities within the catchment riverine systems could potentially impact MP occurrence. It was observed that the main MP pollution sources in the rivers could be WWTP and IA-impacted sites based on the MP abundance data. High levels of MPs were observed in the IA site followed by the WWTP-impacted site. Furthermore, the MP types (fiber, film, pellets) support this finding, as fiber was the most abundant across all sites. These findings indicate that within the uMsunduzi River, the main sources of microplastic pollution are wastewater treatment plant effluent and industrial sites. The comprehensive investigation into MP contamination in the uMsunduzi River has provided valuable insights into the intricate relationship between MP pollution and microbial communities. The identification of various MP polymer types and their abundance highlights the pressing need to address the growing issue of MP pollution in aquatic environments. The presence of distinct microbial communities in MP surfaces distinguishes the plastisphere from surrounding habitats by selectively recruiting microbial communities, thus underscoring the complex ecological dynamics at play.

The major conclusion of this study includes:

This study confirms that different pollution sources significantly impact MP abundance, with IA-impacted site contributing the highest number of MPs (69 particles/L) in surface water, followed by WWTP-impacted site (51 particles/L). The shape and polymer types of MPs released varied across pollution sources. Fibers were the most dominant shape across all sites, accounting for 60% of surface water and 59% of sediment samples, reflecting their prevalence

in effluents and runoff. Key polymers identified PE, PP, PS, PC, and nylon. The WWTP-impacted site was characterized by PE, PP, PS, and nylon, while the IA-impacted site showed predominance in PE, PS, and PP. The UA-impacted site had high levels of PE and nylon, while the AA-impacted site exhibited PE, PP, PC, and PS. These results highlight that pollution sources not only influence the abundance of MPs but also determine the specific shape and polymer types present, underscoring the relationship between human activities, land use, and environmental contamination.

Significant differences in bacterial community diversity were observed between surface water and the plastisphere, with surface water showing higher bacterial diversity than the plastisphere. The dominant bacterial phyla across the various habitats and sites were *Proteobacteria* (30-70%), followed by *Bacteroides* (2-15%) and *Actinobacteria* (2-7%). Although most bacterial species were common across habitats, a unique bacterial community was evident in different environments. Furthermore, the study showed clear habitat and site-specific enrichment of bacterial taxa between surrounding surface water and the plastisphere, with significant seasonal variability. Surrounding surface water consistently hosted more unique (52) and abundant bacterial groups compared to the plastisphere (21).

In total, 17 bacterial pathogens were identified across different habitats (surface water and plastisphere), sites (urban areas, wastewater treatment plants, agricultural areas, and industrial areas), and seasons (summer and winter). The MP surfaces (19) were found to harbor more ARG classes than the surrounding surface water, which contained only 9 ARG classes, suggesting that MPs act as concentrated reservoirs for antibiotic resistance. The WWTP-impacted site exhibited a more diverse number of ARGs confer resistance, including tetracyclines, fluoroquinolones, and aminoglycosides, reflecting the influence of pharmaceutical effluents.

Finally, the QMRA evaluated the potential infection risks associated with *P. aeruginosa* and *S. enterica* due to exposure to surrounding surface water and biofilm-associated with MP surfaces in the uMsunduzi River. The results showed that the presence of *P. aeruginosa* and *S. enterica* on the MP surface significantly increases infection risks compared to exposure from surrounding surface water alone. MPs enhance pathogen persistence, distribution, and exposure, leading to elevated infection probabilities for both children and adults across all sites and seasons. These findings underscore the critical role of MPs as vectors, amplifying health risks in contaminated aquatic environments.

Future Recommendations:

- Further research should investigate the long-term effects of seasonal variations on the accumulation and persistence of ARGs on microplastic surfaces, as this study identified seasonal differences but lacked in-depth temporal analysis.
- There is a need for more comprehensive studies that examine the interactions between different types of MPs and pathogens as well as a broader range of emerging contaminants, such as pharmaceuticals and heavy metals, to better understand the full scope of pollution and its ecological impacts.
- Future risk assessments should incorporate more detailed exposure scenarios, including different water activities and ingestion rates, as well as refine the probabilistic models to improve accuracy in predicting infection risks associated with pathogens on microplastic surfaces. Including multiple pathogen types and broader environmental conditions would also enhance the understanding of health risks.

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