# Study on the Presence of Microplastics In Bottled Drinking Water Using Nile Red and Its Impact on Human Health


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A thesis submitted to the Department of Mathematics and Natural Science in partial fulfillment of the requirement for the degree of Bachelor of Science in Biotechnology


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November, 2024

## Declaration

It is hereby declared that:

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2. The thesis excludes earlier published material or is not written by the third party, perhaps, cited appropriately and referencing accurately.
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## Ethics Statement

We, Tahseen Afroz and Arpita Roy Tithi, hereby certify that the following criteria are fulfilled in the manuscript “Study on the Presence of Microplastics In Bottled Drinking Water Using Nile Red and Its Impact on Human Health”

1. This content is entirely our own work and has not been published before.

2. All sources utilized are properly credited through precise citations and justified references.

# Abstract

Microplastics have emerged as ubiquitous in the environment as a pervasive global concern, alarming to the environment, agriculture, marine ecology, living organisms and human health. Several experimental studies reported microplastics have been comprehensively detected in environmental elements such as ocean, rivers, air, soils everywhere even within drinks and foods. However, packaged plastic water bottles caused microplastic pollution in drinking water in developed countries like Bangladesh, due to restricted information. Defined as minute plastic fragments ranging from 1.5 µm to 5 µm, microplastics pose a potential threat to human health. Initial investigations suggest their existence in bottled water and beverages, with these indigestible particles persisting in the human gut and circulating in the bloodstream, potentially reaching the brain. The chemical composition of microplastics raises concerns about their toxic effects on cells. This study aims to scrutinize the prevalence of microplastics in packaged water nationally and internationally, encompassing mineral water commonly consumed in daily life. The focus is on Polyethylene tetrata identified as targeted microplastic categories. Fluorescence microscopy and FTIR are employed to detect and analyze microplastic characteristics such as size and shape. Preliminary findings from 36 water bottle samples exhibit prominent 36% bead-like structures and 33% fibers and 20% fragments under microscopic analysis. Subsequent research will delve into the toxicity of microplastics on human health. So far, 36 samples of plastic bottled water have been used to detect the presence of microplastic. Therefore, comprehensively, amount of MPP on average using NR dye in acetone solution is 10 µg/ml per sample is (23.17± 7.04) items/filter and the MPP on the average using NR dye in acetone solution is 20 µg/ml per sample is (33.33± 10.4) items/filter through fluorescence microscopic analysis. Moreover, the average MPP in an optical Microscope is (20.42 ±7.63) items/filter through optical microscopy analysis. MP polymers PET is characterized through the FT-IR spectroscopy method.  Therefore, there are on average (69±11) items/litter observed which are in size <1 mm and there are on average (99±11) observed particles which are less than 1mm. The significant difference ($p<<0.05$)  shown in between the shapes and the sizes. The estimation of dietary intake of mp for an adult is 2 particles per liter daily, translating 730 in annually identified through survey report. The ultimate goal is to ascertain the number, type, and size of microplastic particles in plastic bottles, providing insights into the annual microplastic intake by the Bangladeshi population through beverage consumption.

**Keywords:** microplastics, bottled water, human exposure, FT-IR, environment, toxicology.

# Acknowledgement

First, we would like to thank God for guiding us and being with us throughout the journey of Thesis.

We are grateful to Professor DR. Md. Firoze H. Haque, Ph.D., Chairperson of the Department of Mathematics and Natural Sciences, for allowing us to study in this department.

We are indebted to our supervisor, Dr. Munima Haque, who is a Director and Coordinator of the Biotechnology Program in the Mathematics & Natural Sciences Department, for sparing her precious time, inspiring us, making it more simple with guidance, effort beyond words, and patience, as well as offering mentorship and aid from start to end efficiently!

And lastly, we would like to thank our families for believing in us all the time and supporting us no matter what, just encouraging us more than anything.

**Tahseen Afroz and Arpita Roy Tithi**

November, 2024

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## List of Acronyms

MP : Microplastics

NR : Nile Red

FI-IR : Fourier Transform Infra-Red

RM : Raman Spectroscopy

SDS : Sodium Dodecyl Sulfate

EDTA : Ethylenediaminetetraacetic Acid

WHO : World Health Organization

DDT : Dichlotorodiphenyl-Trichloroethane

Al : Aluminum

PE : Polyethylene

PET : Polyethylene Terephthalate

PVC : Polyvinyl Chloride

HDPE : High Density Polyethylene

LDPE : Low Density Polyethylene

UV : Ultra-Violet

# Chapter 01

## Introduction

Microplastics, the word explains that they are plastics in micro sizes. These tiny particles of plastics are principally, the fragments of large plastic particles. These minute particles, often less than 5 mm in size, originate from a variety of sources, including personal care products, cosmetic products, synthetic textile industries, car and tyre manufacturing industries, color and paints. These sources can intentionally produce microplastics for their own benefits, on the other hand, microplastics can be generated by degrading the primary products of the mentioned sources. Based on that, they are categorized in primary microplastics and secondary microplastics. The primary microplastics are the intentionally-made microplastics, like microbeads, microfibers etc. in contrast, secondary microplastics are the result of disintegration of larger plastic particles. The ubiquity of the use of plastics has now led to the development of microplastics, which are now polluting the environment, from the deepest oceans to the highest mountains. Not only that, microplastics are now found inside the living organisms which is elevating the concerns.

The detection of microplastics has become crucial due to their widespread presence in the environment which is causing potential risks to ecosystems and human health. Different research teams have used different materials to detect the presence of microplastics on different samples. To detect the presence of microplastics on consumable freshwater, the most beneficial method is to use fluorescent dye, named Nile Red. Nile Red is a hydrophobic dye that readily binds to hydrophobic particles and allows visualization and quantification of microplastics under fluorescence microscope. As microplastics involve potential risks to environment and health, along with detecting the presence of microplastics, understanding their behavior and effects is also necessary. More sophisticated techniques for understanding microplastics are Raman Spectroscopy, FTIR spectroscopy etc. which provide specific and sensitive information of the samples. Since the amount of microplastics pollution is rising, the prevalence of finding them on every sphere of the environment is also expanding alarmingly. So detecting microplastics on the packaged drinking water is also a significant concern as they are being consumed all the time. So it is urgent to address the issue of microplastics pollution and regulate the production of plastics and microplastics to save the earth and its living beings.

# Chapter 02

## Microplastics Production

## 2.1 Introduction

In the modern era, the most concerning matter is the rise of plastic pollution. Plastic was first invented in the 20$^{th}$ century and till now, traces of first generation plastics can be found on the earth. Plastics are made by polymerization which induces a strong bond between the molecules and makes it difficult for microorganisms to decompose the plastic particles. As a result, plastic remains in the environment unless they are recycled or reused. The plastic particles that remain in the environment can then break down into smaller particles due to various environmental and mechanical forces. These smaller particles, ranging from 1 to 1000 µm can be classified as microplastics. The production and accumulation of microplastics is a complex issue because it is affecting both wildlife and humans, and in a developing country like Bangladesh, production of plastics and microplastics are increasing notably. Uncontrolled production of plastic products, improper management of plastics disposal, extensive use of plastic products in daily life and littering anywhere contribute to the production of microplastics.

## 2.2 Production of Plastic and Applications

Beginning the accomplishment story of the latest group of materials, plastics have been started by the middle of the last century which has excellently modernized human daily life and introduced the starting of "plastic age" (Thompson et al. 2009). Nowadays, people prone to use plastics satisfactorily due to its durability, flexibility and waterproof ability, however its uses threatened environment (e.g. water pollution, soil pollution, air pollution) as well as human life (Laist 1987; Cole et al. 2011; Wright et al. 2013). One of the anthropogenic issues is plastic pollution which is accumulated by the plastic substance including particles and objects in the environment that unfavorably affect human health, survival of population and wildlife habitat (SB & AA 2020). The production of plastics has dramatically increased which is 367 million tons in the year 2020 (PMERG 2021) and 390.7 million tons in 2021 (PMERG, 2022). Plastic pollution can be classified based on many aspects, such as chemical and physical properties, types, ranges and including divided in various sizes such as nano debris plastics (0.001–1 µm), micro debris plastics (1–1000 µm), meso debris plastics (1–10 mm) macro debris plastics (> 1 cm) (Table.1.1) (Bermúdez & Swarzenski 2021; Morioka et al. 2023). Therefore, the impact of the microplastics in marine ecosystems, aquatic environments especially, seas, oceans, water bodies, and surface water and groundwater increased over the hundred years where the microplastics are moved all over the world via oceans and found in bulk on the surface. These particles released hazardous organic pollutants as DDT (dichlotorodiphenyl-trichloroethane) and were consumed by the fish, birds and many marine animals (Gore & Kandasubramanian 2018; Issac & Kandasubramanian 2021).

Plastics are long chains and high molecular weight, made up of synthetic or semi synthetic polymers with the properties of thermoplastics or thermoset plastics (table 01) (Desidery & Lanotte, 2022). Thermoplastics have a unique feature as they have lower melting point (e.g. 110˚C to 175˚C) and become softened when they are exposed by the heating-cooling process of repeated cycles with less degradation. Due to its long shelf-life, low moisture absorption, flexibility and durability, the production of the thermoplastics and uses in the form of diverse products are the cause of several pollution (Alauddin et al. 1995; Pucci 2018; MacLeod et al. 2021). PET, PTFE, PP, PS, PE, HDPE, LDPE, PVC are the most common types of thermoplastics, and widely used for various products such as medical equipment, bottles, cosmetics, agriculture lines, packaging, toys, electronic assembles and so on (Chen et al. 2023). Moreover, globally the production of PE fiber stood 63.3 million in 2022 (Statista, 2023), LDPE production stood 820000 metric ton in the United State of America in 2021 (Statista, 2024b) and HDPE production stood 4.16 million metric tons in China (Statista, 2024). Thermosetting plastics are defined as solid polymers because they do not melt easily once done curing, forming high molecular weight 3D structure and becoming solidified due the bond between cross molecules is complex inter-connected which is irreversible breakdown (Alauddin et al. 1995). The common varieties of thermoplastics are epoxy resins, silicon, polyurethane and so on (Alauddin et al. 1995, Alarifi, 2022).

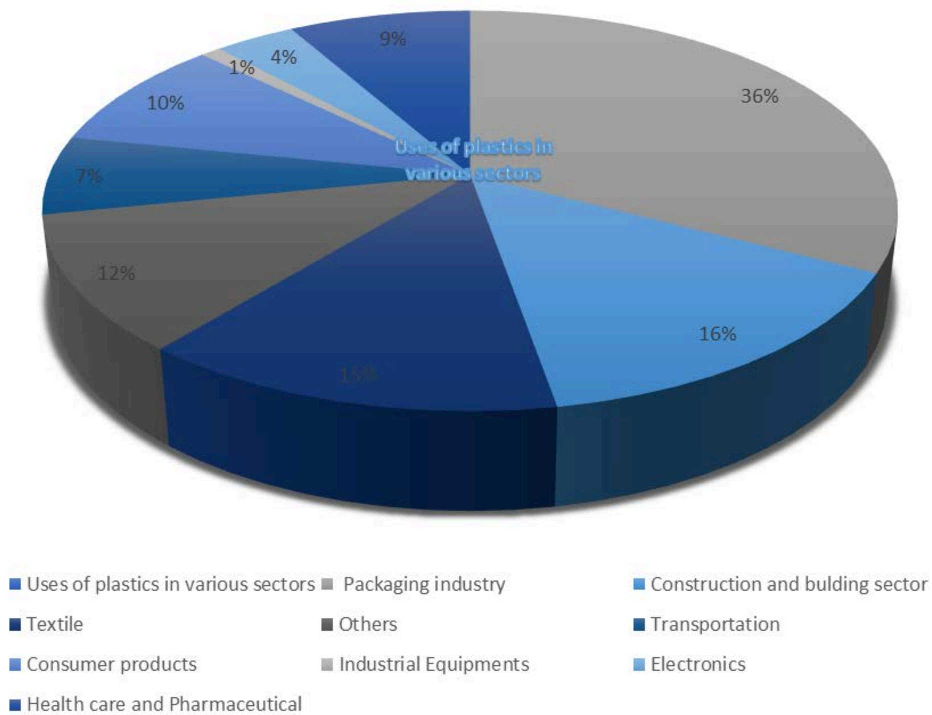
In this modern era, it is unimaginable without plastics as people widely use of plastics in industrial and domestic purposes for its several benefits such as cost effectiveness, different varieties of applications, light weight, long-lasting and flexibility and so on (A et al. 2019), however the first widespread use of synthetic plastic commercially available in the early of 20$^{th}$ century which is Bakelite. (Geyer et al. 2017). Depending on the properties of physicochemical the manufactures, productions and uses can be divided in many sectors (figure 1.1). The most dominant sectors of using plastics are packaging industry, construction and building sector, textile and so on. Moreover, the use of plastic is increased in the packaging sector which is 36% and uses PET, LDPE and HDPE most (Scalenghe 2018; Muneer 2021). In 2015, the most plastic produced in United State of America is 42.8 MMt (Kan et al. 2023) where they used 34.8 and the second largest country China produced and used plastics more than any other Asia countries where 16 million were recycled (Plastics Europe, 2023; Kan et al. 2023; GreenMatch.co.uk, 2024).

## 2.3 Plastic Pollution in Developed Country Like Bangladesh

Bangladesh is already one of those developing countries which are on the rise and high plastic item usage in this country provides a major threat to biodiversity and environment as shown in figure 01. Bangladesh also banned the manufacturing, importing and selling of polyethylene bags which are below 55 μm in thickness since November, 2002 for its hazardous impact on opposite living creatures due to plastic pollution according to Bangladesh Environment Conservation Act No.1 without year. Due to satisfactory output of plastic bags, people are prone to use plastic bags instead of jute packaging materials. Moreover, plastic waste was only 1.74% of the total garbage in Dhaka's landfills back in 1992, and this figure has since increased up to approximately 7.5% with respect to Plastic waste generation by overall other types until end of year 2019 based on Statistical facts. In Bangladesh, 8% of the annual waste produced is plastic. In numbers, this equals 800,000 tonnes. Consequently, 14 million fragments of a polythene bag are used every day inside Dhaka and they often find their way into rivers that threaten marine life. Along with that, the Padma, Jamuna and Meghna rivers also alone pour around 73,000 tonnes of plastic debris into the ocean daily. (Bangladesh Waste Database 2021, 2021).

Above all, so many of them keep killing the rivers and lakes along with tanks; as a result they share their density in pollution levels which hugely contributes to biodiversity loss at Bay Of Bengal. Furthermore, microplastic pollution will pose a serious threat to human health and the environment, can be harmful and poisonous for marine life in addition to being widely distributed in the food chain that feeds humans and other animals (Muhammad et al., 2021; Hossain et al. 2023).

**Figure 01: The Percentage of Plastics Used in Different Sectors.**




However, the increasing trend of plastic in urban garbage could be an alternative for GDP growth that mirrors soaring use throughout the emerging country as Bangladesh. The rise in plastics poses problems for Bangladesh, turning possibilities into opportunities. Practicing source separation and resource recovery can turn plastic trash into another valuable fuel in the shape of refuse-derived fuel pellets. As a fuel, now in Bangladesh, they have started developing and producing Refuse-derived fuels by most of the wastes like plastic, paper, cardboard fabric & textile waste that could potentially be used as an alternative method for using those materials. This research is at an experimental stage right now (Bangladesh Waste Database 2021, 2021) .

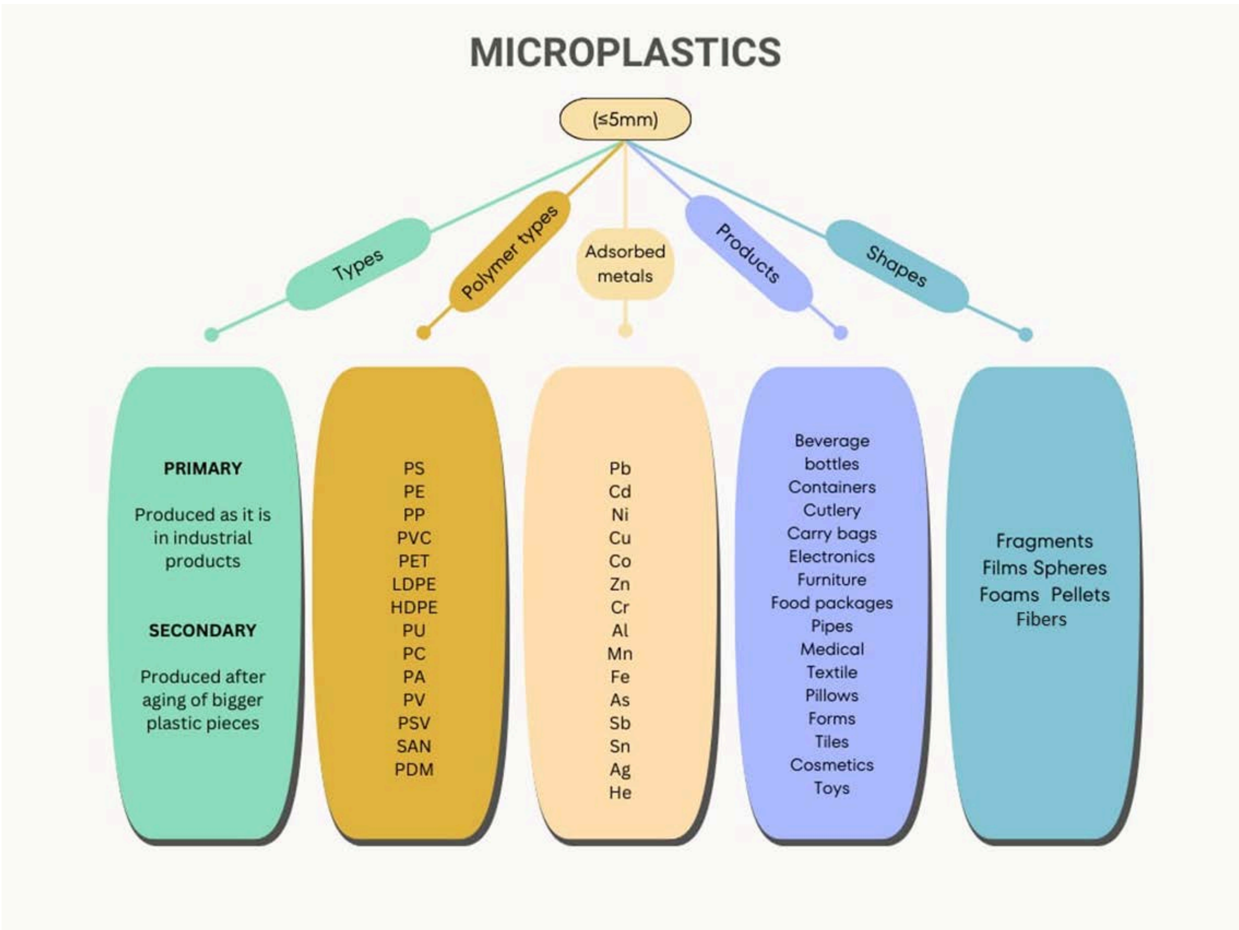
## 2.4 Production of Microplastics from Plastic Particles

Plastic contamination in various natural surroundings can be seen worldwide which is remarkably recognized as a significant concern for scientific researchers and general audiences (Eerkes-Medrano et al. 2019; Novotna et al. 2019; Mintenig et al. 2019; Shen et al. 2021). Plastics in different types, sizes and forms float in water bodies, including, freshwater, ocean, sea, river, lake and so on which are contact directly to the sun, react to chemical elements such as oxygen and deterioration from physical impact by waves and sand factors for breaking down smaller pieces, mentioned many researchers and studies as 'Microplastics' (Gregory, 2009).

## 2.5 Definitions and Characteristics of Microplastics

Microplastics are smaller pieces, water insoluble, synthetic solid polymers that are 1µm to 5µm in sizes observed mostly in marine environment (Bergmann et al. 2015; Lehtiniemi et al. 2018; Pivokonsky et al., 2018; Eerkes-Medrano et al. 2019; Koelmans et al. 2019; Shen et al. 2021), despite that there is debate to the definition. According to the Technical report ISO/TR 21960, water insoluble plastic shreds in any dimensions, the sizes within 1µm to 1000 µm refers to microplastics (figure 02), while the term 'large microplastics' is used for the sizes in between 1-5 mm. Nevertheless, the California State Water Resources Control Board states that the chemically unmodified three-dimensional plastic particles which are greater than 1 nm and less than 5000 µm are referred to as 'microplastics'. Therefore, based on the sizes the plastics nomenclature can be different such as nano-plastics, sub-micron plastics, small or large microplastics and meso or macro microplastics and so on (ISO/TR 21960:2020, n.d.; Board, n.d.; Koelmans et al., 2015.;Schymanski et al., 2021, Bermúdez & Swarzenski 2021; Morioka et al. 2023).

**Figure 02: Diversity of microplastics along with the absorbed heavy metals, products and their shapes.**




All of these microplastics are non-biodegradable because of their chemical composition. The long chain of carbon and hydrogen molecules induce a strong bond and have no enzymatic activity, thus they are not feasible for microorganisms to degrade the particles.

## 2.6 Types of Microplastics

Microplastics are classified by origin as primary plastics, manufactured indigenously in smaller pieces while the asymmetric fragments, beads, pellets are originated from larger plastics products or objects. These excessive variation form, size, and colorful types of polymers surrounded by secondary microplastics (table 01) float in the marine environment, since they can result from disruption of larger pieces or plastic products that are produced from plastic packaging including abrasion from bottles, caps and labels are degraded due to ultraviolet radiation, chemical and physicochemical properties as well as biotic factors. On account of, all over the places, the smaller micro plastics spread physically and chemically in numerous organisms in pelagic zones and benthic habitats of marine environment (GESAMP, n.d.; Foekema et al. 2013; Mathalon and Hill 2014; Lehtiniemi et al. 2018) and they have ability to absorb organic contaminants and heavy metals (Karami et al. 2017; Pivokonsky et al., 2018; Hossain et al. 2023). Thus, the toxic elements of microplastics, for instance, tiny plastic particles, plastic additives and contaminants may clot or abrasion the ingestion of the organisms to the human body (Zhang et al., 2021; Hui. et. al., 2021; Wang et al., 2021; Hossain et al. 2023).

Microplastic contamination is an ubiquitous issue that affects not only marine environment but also in human health highlights through many scientific researchers in scientific publication (Yang et al., 2023; Lee et al., 2023; Hossain et al. 2023). Chemical exposure from decomposed used plastics or plastic materials (e.g. bottles) may cause endocrine disruption (Fossi et al., 2012 ; Andrady, 2011) and found in bloodstream, lung tissue (Amato-Lourenço et al.,2021;Li et al., 2023) . Indeed, scientific studies reported the existence of microplastic from every place in the world, even from human placenta (Dekiff et al., 2014; Fok & Cheung, 2015;Wu et al., 2019; Radisic et al., 2020; Muhammad et al., 2021; J. Zhang et al., 2021; Hossain et al., 2023). World

Health Organisation (WHO) states three ways to impact public health that are smaller particles of microplastics can be ingested or inhaled quickly and easily, through micplastic's chemicals such as absorbed moieties, additives and these particles can act as carrier for microorganisms to colonized resulted biofilm formation (Singh et al., 2022). Therefore, the primary and secondary microplastics contaminated freshwater sources and bottled water. Drinking water bottles made from PET, PP AND PE (Table 01) disperses more than 100 µm and 10.4 mp particles approximately per litter (Singh et al., 2022).

**Table 01. Thermoplastics and Melting Temperature:**

| ***No.*** | ***Thermoplastics*** | ***Temperature ˚C*** | ***References*** |
|---|---|---|---|
| ***1.*** | *PE* | *105˚C - 135˚C* | *(Polyethylene, n.d), (Eu* |
| ***2.*** | *LDPE* | *105˚C - 115˚C* | *Fernández and Puig (2002b), Royan et al. (2021).* |
| ***3.*** | *HDPE* | *120˚C-190˚C* | *Fernández and Puig (2002b), Royan et al. (2021).* |
| ***4.*** | *PP* | *160˚C – 170˚C* | *Jindal (2024), Federation (n.d.).* |
| ***5.*** | *PET* | *235˚C- 260˚C* | *Fernández & Puig (2002), Royan et al. (2021)* |
| ***6.*** | *PS* | *240˚C – 260˚C* | *Habib (2023), PlastikCity (n.d.)* |
| ***7.*** | *PVC* | *100˚C – 260˚C* | *Root (2023), Jindal (2023)* |

# Chapter 03

## Sources of Microplastics

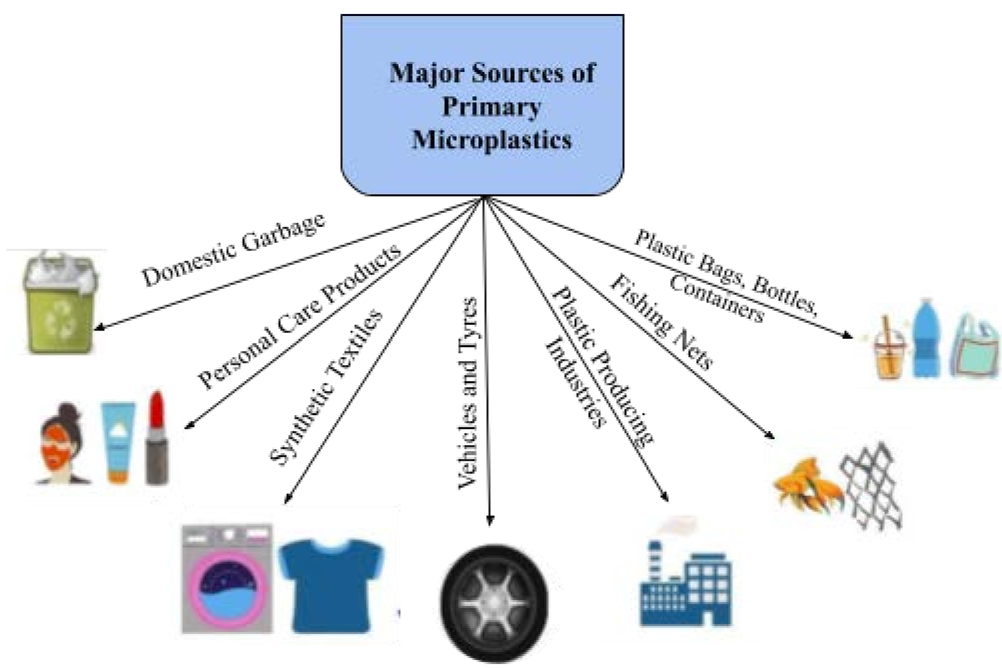
## 3.1 Introduction

Microplastics are the most hinder for aquatic life. According to the UNESCO Intergovernmental Oceanographic Commission report, each year, around 8 to 10 metric tons of plastics enter the ocean, and in total, the number of microplastics pieces is over 50-75 trillion (Fava, 2022). Plastic particles are manufactured by using polymerization reactions and the chemicals that are used in the process are toxic to the environment. In addition, most of the plastics present on the earth are non-degradable. Thus they stay on the water for years and disturb aquatic life. Fragments, fibers, granules, pellets of industrial plastics are the most common microplastic distinctions found on the water bodies. Microplastics can mix with the ocean water from various sources like textile industries, car tires, plastic pellets, personal care products etc. Microplastics can even be produced by breaking down large plastic particles into smaller fragments. Although most of the plastic particles end up in the ocean from the land, it is difficult to identify the exact source of microplastics in the water. It can be produced in micro size or it can be a fragment of other plastic particles. Hence, based on the shape and size of the microplastics, they are classified in two major categories, the primary source of microplastics and the secondary sources of microplastics.

### 3.2 Primary Microplastics

Microplastics are divided into two major categories according to their production procedure, primary microplastics and secondary microplastics. The primary microplastics, in simple terms, are the plastics that are produced in micro sizes for industrial purposes. These microscopic synthetic polymers are no more than 5 mm in measurements. The most prevalent primary microplastics are microbeads, microfibers, plastic pellets and glitters (*Microplastics*, n.d.). A large number of these plastics are used in the cosmetics industry. Personal care products like exfoliating face washes, toothpastes, and makeup products like mascara, eyeshadows, lipsticks contain microbeads and glitters. Synthetic textile industry is most likely to produce microfibers, namely nylon and polyester. These popular plastics are produced in micro sizes for clothing preparation and later they shed tiny fibers while washing. Drying and tearing of the materials can also contribute in primary microplastics production, to be more specific, microplastic contaminations in the environments. Industrial granules or plastic pellets are another source of primary microplastics. These microplastics are commonly known as nurdles that are the raw materials of plastic products. During transportation and handling the nurdles, they can be released into the environment and the toxic chemicals of the nurdles can affect the ecosystem. Each year, around 445970 tonnes of nurdles accumulate in the environment (Phillip, 2024).

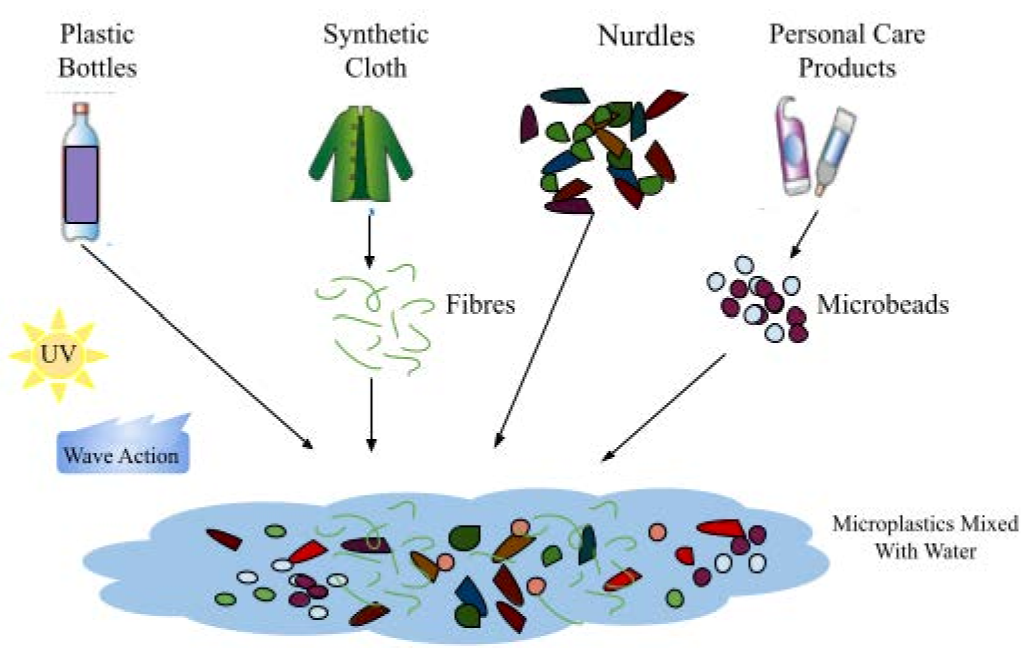
**Figure 03: Primary sources of microplastics.**



### 3.3 Secondary Microplastics

Secondary microplastics derive from larger plastic particles. They are usually the results of environmental exposure of the plastic materials. Researchers found that most of the plastic pollution is caused by littering and the majority of the litter includes plastic wrappings, bags, bottles, razors etc (Fava, 2022). As these are not disposed properly, it ends up exposed in the sunlight. Also in beaches, tourists throw away their trash in the sands which later end up in the ocean. All these events lead to the production of secondary microplastics shown as figure 04. The ultraviolet rays radiate from the sunlight and make the plastic polymers brittle. Moreover, the waves and winds in the seashore contribute to the fragmentation of plastic particles. As they take hundreds of years to decompose, they remain in the environment for a long time and can be consumed by animals.

**Figure 04: Production of secondary microplastics from primary microplastics**



### 3.4 Common Sources of Primary and Secondary Microplastics

There is no place in the world where plastic particles do not exist. The chemical bonds in the plastic particles are strong enough to resist the natural degradation process. Instead of degrading, these plastic particles break down into smaller particles due to environmental factors. In contrast, some of the microplastics are intentionally manufactured for numerous products. Microbeads are profoundly found on personal care products. Toothpastes, facewash, body wash, scrubs etc. often contain microplastics as they are considered as exfoliator and texture enhancer. These microplastics are in minute size and drained through the wastewater. Textile industries are the manufacturer of synthetic microfibers. On the other hand, packaging industries are responsible for producing plastic wrappings, plastic bottles, containers and so on. Painting industries also contribute to microplastics pollution as plaints use microplastics to enhance the pigmentation and durability. With time, these paints and coatings leach into the ground and later mix with the water systems. All of these items can deteriorate into smaller pieces in the influence of the environment, specifically for sunlights and waves.

# Chapter 04

## Microplastics Pollution in Environment

### 4.1 Introduction

The presence of microplastics in the environment poses significant challenges to both wildlife and human health. The use of plastic particles is increasing day by day and most of them are non-degradable. Although bioplastic is present in the market, it has its own drawbacks. Bioplastics are specifically plant-based plastic, so it requires a huge number of plants. As it is not feasible for a long run, most of the plastic products are made with petroleum-based polymers. These polymers induce a strong bond between the carbons and make it non biodegradable. As a result, in the environment, the amount of plastic particles is escalating significantly. Moreover, the plastic particles are breaking down into smaller particles, namely microplastics in the influence of environmental elements. Along with landfills, annually, a lot of plastic products, including bottles, containers, toys, bags, razors, utensils etc. are collected from oceans, rivers and lands as well. These plastics can leak microplastics, and as the size of microplastics ranges from 1-1000 μm, it can easily pass through wastewater sieves and can mix with running water. Similarly, those microplastics can get mixed with the soil which can result in the alteration of soil properties, The chemical compounds of microplastics can induce toxic effects if they are consumed by any aquatic or terrestrial animals. From them, microplastics are now introduced in the human body that are causing difficulties.

### 4.2 Microplastics Pollution in Marine Ecosystem

The presence of microplastics in the marine ecosystem is a major concern because of the potential harm they induce in the environment. Traditional plastics can be found on each product we use everyday. Since most of them are not biodegradable, they somehow end up remaining in the environment. These include polyethylene, polypropylene, polystyrene, nylon (Barboza et al., 2019) in the form of microfibers and microbeads. From the lands, the plastic litter can float to rivers and then onto the ocean. Several studies have detected the microplastics in ocean water and microfibers were abundant in each sample. In the NE Atlantic Ocean, around 96% of the microplastic particles were microfibers. In the Irish Continental Shelf, the percentage was 85 for microfibers. Sample of various locations, including Mediterranean Sea, SW Indian Ocean only had microfibers in the water (Coyle et al., 2020). Another recent study on the Tropical Eastern Pacific and Galapagos archipelago specifically mentioned that these microplastics were present in sedimentary habitats, shores, deep sea and in living organisms (Alfaro-Núñez et al., 2021). Microfibers were abundant plastic particles in this ocean as well. The microplastic particle sizes ranged from 150 - 5000 µm and all the size ranges were also noticed in 100% of the samples. These microplastics were even detected in the digestive tracts of marine organisms of human consumption. Among the samples, 71% of them had microplastics on them, which is also higher than previous studies. These microplastics become part of the food chain in the aquatic environment. In basic terms, phytoplanktons can uptake microplastics and then the microplastics can transfer to the phytoplankton-consuming zooplanktons. These zooplanktons can be ingested by macro-zooplanktons and in this way, the microplastic can contaminate the whole food chain.

### 4.3 Microplastics Pollution in Terrestrial Ecosystem

Although the presence of microplastics in the ocean is widely studied, the study is not enough for the land. From the studies on marine systems, the sources of microplastics were determined. Both primary and secondary sources can contaminate the lands. Additionally, products like agricultural fertilizers, sewage sludge, pesticides etc. can pollute the soil. The topsoils near the roads and industries, namely textile, have more microplastics than other areas (de Souza Machado et al., 2018). According to Zeb et al., 2024, these plastic particles can cause chemical reactions in the terrain and alter the soil properties. Research of de Souza Machado et al., 2018 reported that phthalates and bisphenol A are two plastic additives that can leach in the soil. Since microplastics are non-degradable, these additives then interact with the microorganisms of the soils. For instance, microplastic additives can alter the behavior of earthworms if they consume microplastics instead of foods. Microplastics can insert to the plants from the soils and later can end up in the human body. The additives mimic the estrogenic activity inside the human body and can change the hormonal activity in both genders. A very recent detected microplastics in the gut and brain of bees (Al Naggar et al., 2024), and in 2013 Liebezeit & Liebezeit found microplastics on both honey and sugar. The number was 560 microfibers and 540 fragments per kilogram of honey, which would be much higher in recent times. In addition, microplastics were found on the gastrointestinal tract of 94% dead terrestrial birds in the city (Zhao et al., 2016). The case was similar for birds that live near freshwater. Birds consume the microplastics that are smaller than 1 μm with their food, the chemical effects on their gastrointestinal tract results in reduction in number. Similarly, microplastics were detected in different parts of the human body including breast milk (Ragusa et al., 2022), placenta (Garcia et al., 2024), testicles (Hu et al., 2024), heart (Yang et al., 2023), and livers (Horvatits et al., 2022). So it can be concluded that microplastics are not only affecting aquatic ecosystems but also the terrestrial ecosystem.

### 4.4 Microplastics Contamination in Freshwater Sources

Microplastic is ubiquitous in the environment, researchers have found microplastics in every form of water including ocean water, lakes, rivers, wastewater, even on the glacier surface of

Tibetan Plateau. In recent times, microplastic contamination became a concerning issue for researchers because they have found microplastics in freshwater. Only 1.2% of the water in the whole earth is freshwater and now it is also contaminated with microplastics.

Different studies around the globe have found microplastics in different freshwater sources. In the sediment of the Kelvin river, situated in the UK, researchers found nearly 296.5 units of microplastics in each liter of water. Sediment and surface water of the Carpathian basin contains 524 units of $L^{-1}$ microplastics. Vembanad lake of India contains 300 units of $L^{-1}$ microplastics in its sediment (Li et al., 2020). According to WHO report, microplastics enter freshwater sources through surface run-off, from wastewater effluent, industrial effluent, degraded plastic wastes etc (World Health Organization, 2019). Microplastic particles can run-off from lands to rivers, lakes and other water bodies. At the same time, microplastics of the air can get mixed with water and contaminate the freshwater as well. These microplastics can impose potential hazards in living beings because microorganisms can use the microplastics surface to create biofilms. A small number of biofilm forming microorganisms are pathogenic, yet consuming microplastics surrounded by microorganisms can cause adverse effects on living organisms.

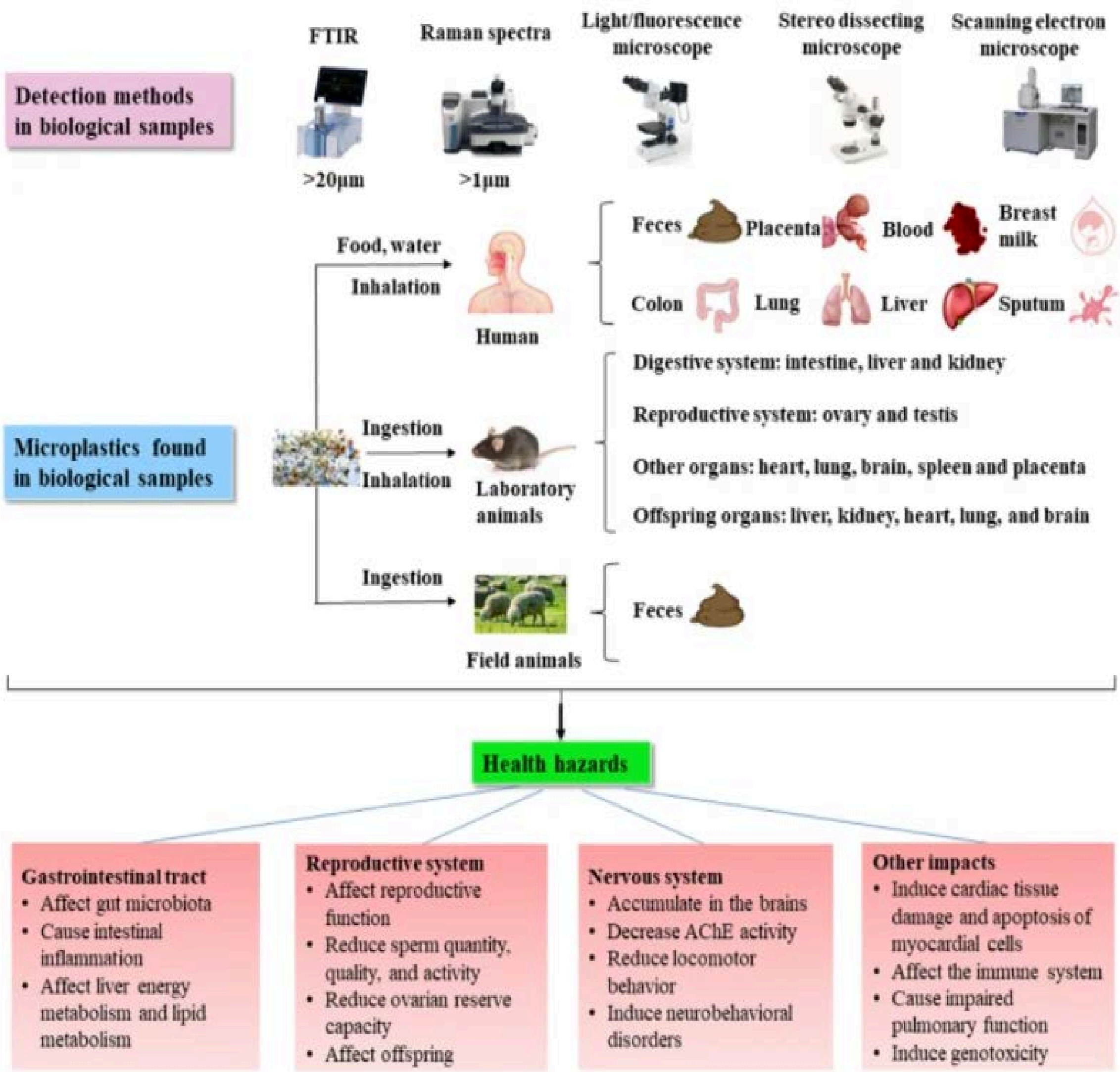
# Chapter 05

## Impact of Microplastics on Health

## 5.1 Introduction

Microplastics are now found everywhere including the living organisms causing it being a growing concern for health. Because of its size, it can easily be consumed by living organisms and also it can remain there if not excreted properly. Studies have found that microplastics are now present in the blood, gut, feces, liver, kidney, brain and even in placenta and testicles. They can insert the body through inhalation also which can cause the microplastic particles to reside in the lungs. Although the health hazards are not fully understood, it deals with many peripheral health issues. For instance, microplastics can accumulate in the bloodstream and can transfer to any part of the body. Microplastics in the testicles can alter the sperm count that can raise the reproductive issue. Similarly, microplastics aggregation on brain cells can influence the alteration of cognitive behavior. Apart from humans, wildlife is also attacked by microplastics. Contaminated water sources, crops, microfiber plastics of the air are contaminating the pollen and eventually the bees and other pollen-dependent living beings shown in figure 05. Therefore, a clear insight of microplastics pollution and impacts of it need to be investigated properly.

**Figure 05: Impact of microplastics on different organisms of animals**. (This graphical abstract is reused here with the permission of the author Liu et al., 2023. The agreement is acknowledged at the end of the report.)



### 5.2 Potential Health Risks to Animal's Life

The chemical composition of microplastics can affect the animals if they are introduced inside the living organisms. In the marine ecosystem, microplastics are often found inside the microorganisms and animals, including phytoplanktons, zooplanktons, fish, whale, turtle, seagull etc. Microplastics enter their body when they mistakenly swallow microplastic particles instead of their food. In addition, microplastics can insert the marine food chain if zooplanktons ingest them. These ingested microplastics can either remain inside the zooplankton or come out from the body as excrete. None of them are harmful as microplastics will not degrade readily after coming out of the zooplankton's body. Similarly, if the microplastic remains inside the zooplankton, it will reduce the appetite of the microorganisms and perform chemical reactions inside the body. Since the chemical components are toxic, it will affect the zooplankton. On the other hand, zooplanktons are consumed by fish and other animals. If anyone consumes one of those aquatic animals, microplastics will enter the human body from them. Similarly, microplastics are affecting honey bees which causes the presence of microplastics on honey and sugars. Soil is contaminated with microplastics as landfills are filled with tons of plastic products. In the same way, in developing countries like Bangladesh, people throw away their trash anywhere which results in the pollution of plastics and eventually, microplastics. The microplastics can alter the soil properties, and at the same time, can be consumed by organisms living in the soil. For example, earthworms are the best creatures for maintaining the properties of soil since they direct the aeration process of the soil. Also they contribute to the nutrient cycle between the soil and other plants. These microplastics can enter inside the earthworm through ingestion, and they are often found together with heavy metals (Cui et al., 2022) and the chemical components of the microplastics and heavy metals will cause toxic effects on them, however, the earthworms will lose its characteristic and gradually the behavior of soil will also change.

### 5.3 Potential Health Risks to Human Life

It is probable that plastic particles are ubiquitous in the environment and they can affect every living being through their diet, or simply by inhaling and passing through the skin. The laboratory animals were tested for identifying the effects of microplastics on the living cells. The reactions included inflammation, immune response, disruption of endocrine cells, modification of metabolism process etc (Kannan & Vimalkumar, 2021). Moreover, microplastics release additives, mainly bisphenol A, phthalate, benzothiazoles, organotins and heavy metals  are more concerning for health because they can disrupt the cell and interrupt with the nerve cells and reproductive cells. Although microplastics only can enter the body via skin after any wound, these three are most likely the key routes of human exposure to microplastics.

#### 5.3.1 Ingestion of Microplastics:

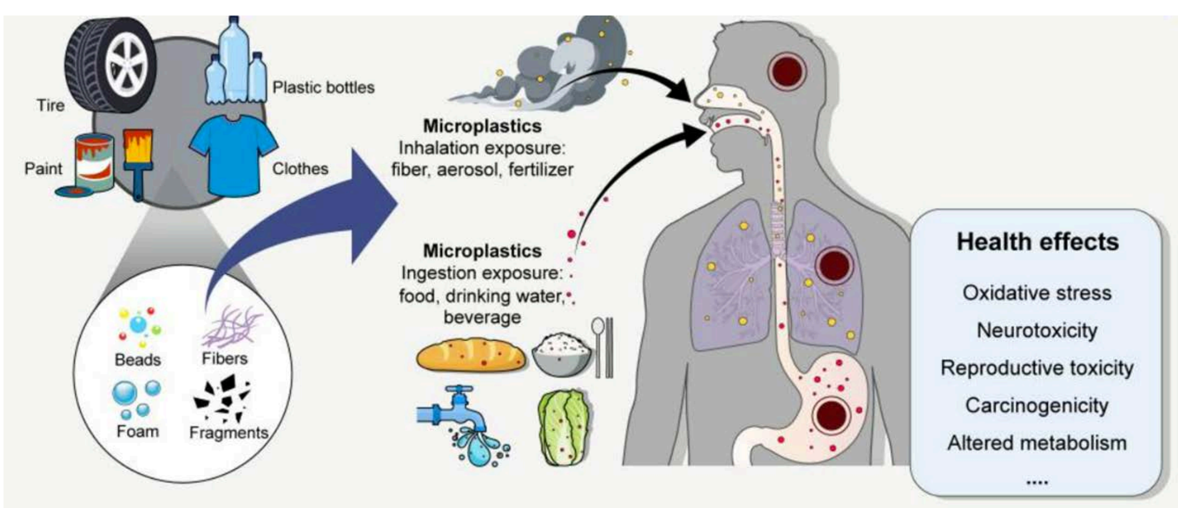
Microplastics can easily contaminate the water supplies and food chain by adulterating aquatic and terrestrial environments. Researchers have observed that microplastics are present in foods like sugar, honey, beer, water, salt, fish, and so on (Kosuth et al., 2018).

Several studies show the statistics where it was found that the average level of microplastics on seafood is 1.48 particles/g, on sugar is 0.44 particles/g, bottled water is 94.37 particles/L, and on air is 9.80 particles/m3 (Cox et al., 2019), and on average, people consume around 39000 to 52000 microplastic particles per year which indicates that human mostly consume microplastics via ingestion through foods and water. (Yee et al., 2021). Consumed food enters the gastrointestinal tract. Microplastics can not proceed the paracellular transport due to its size, so the plastic particles usually reside in the gut lumen (Alberts et al., 2002). These xenobiotics possibly can enter the lymphatic system and are attacked by the immune system.

### 5.3.2 Inhalation of Microplastics:

Similar to ingestion, microplastic particles can invade the body by inhalation. Synthetic textile industries can produce lightweight microplastic fibers that can drift through the air and be inhaled by any of the living organisms. Due to the high surface area, plastic fibers can be stuck on the alveolar surface and can lead to lung damage due to its chemical toxicity shown in figure 06, also high exposure on contaminated air can enhance the risk of inflammation to lung cancer (Prata, 2018)

**Figure 06: Health effect of microplastic exposure. (**This graphical abstract was reproduced from the open accessed articles of Lee et al., 2023.)



# Chapter 06

## Literature Review

## 6.1 Microplastic Pollution in Water

Microplastics pollution can be the reason for marine litter from incaution discarding of waste products which either straightly or laterally end up into lake, sea or ocean. Wastes produced from plastics found throughout the Arctic to Atlantica's ocean in surface and ground water,on shorelines and bottom of sediment. They may congregate close to significant sources, shipping channels, inhabited places, and remote locations like mid-ocean gyres.

Andrady (2011) broadly categorized the plastics into three in terms of their chemical composition: PP, PE and PS which were known as polypropylene, polyester, polystyrene. This work examines the fate of plastics in general and the pathways through which microplastics are introduced to ocean environments making PET, PVC are considered as proxies for most forms of plastic. Exposure of these plastics under the sun resulting in UV radiation and other natural forces such as waves and tides would lower the average molecular weight. These are then brittle enough for the plastics to shatter into powdery fragments, called microplastics. This method also causes plastics to be leached with chemicals into the water, in turn rendering the ocean more toxic.

According to the review, the marine biota could ingest highly persistent organic pollutant microparticles. Being the largest and deepest enclosed sea in the world, located at one of busiest intersections for navigation globally and both needing to cope with thousands of millions visitors per year as a top tourism destination, it is no wonder that human activity has more impact there than anywhere else. Its heavily populated coastline, restricted surface water outflow and well developed activities of fishing, shipping, tourism and industry say the global model has calculated that more floating plastics will concentrate in the Mediterranean Sea than anywhere else on Earth. Consequently, huge amounts of marine litter tend to get stacked up in the basin.As a result, given the growing oceanic plastic pollution, a better understanding of how microplastics affect marine food webs is urgently needed.

Eriksen et al. (2013) presented that it is undeniable that not only marine but also freshwater environments can be contaminated with microplastics. This study observed the initial survey of open-water plastic pollution in the Laurentian Great Lakes system. Samples were collected at 21 locations across the three lakes such as Lake Superior, Huron and Erie taken for scanning electron microscopy examination. The exception of plastics found in all stages of production where the average abundance was 43,157 particles/km$^2$ using Scanning Electron Microscope. Along with that, Aluminum Silicate particles detected in  coal or fly ash presented among eight locations from 21 locations with an average contribution. The size range of these particles  was between 0.355 to1 mm and  roughly 20%. The particle sizes detected in Lake Erie samples were from 0.36 to 0.99 mm, and accounted for nearly 85% of the total microplastic particles collected across all samples participated between lakes combined. Among the five microplastic type groups that were detected, pellets and fragments contributed to 81% of total particles. Scientists identified the Great Lakes and also specific particles  in eight states that are home to more than 144 coal-burning power plants which may discharge wastewater into rivers containing aluminum silicates. In freshwater systems, the multicolored spherical particles seem to stem from consumer goods like face cleansers and other personal care items.

Faure et al. (2015) The study analyzed all together with these 39 surface samples, which were gathered with a 300µm-mesh plankton net, the research's main goal was to increase our understanding of microplastic contamination in Swiss surface waters in order to better understand its distribution, destiny, and effects. Using a stereomicroscope for visual inspection, the samples revealed microplastics in all of them at amounts that varied between 11,000 to 220,000 particles/$km^2$. After performing a particle size segregation, the resulting particles were further divided into several groups on an optical table according to their shape and properties. Pieces made up the majority, with pellets and foams making up a minor portion. The FT-IR analysis of the soil's composition followed by an ATR spectroscopy result showed that the majority of the polyethylene was the product of EM-polymerization following Py-GC/MS. These were found in significant quantities (over 62 percent) for packing purposes; polypropylene (15 percent), roughly in the form of fragments, and polystyrene (12 percent), roughly in the form of construction materials for insulation, were next. The abundance and frequency of micro plastics in freshwater environments are confirmed by the current investigation.

Mani et al. (2015) provided information on the amount and variety of microplastics that are found at the surface of the Rhine River, one of the largest rivers in Europe. Using 31 samples from 11 different places, the scientists covered the whole 820 km of this river and discovered microplastics in all of them, albeit in variable levels. These samples all included a range of sources and sinks, such as industries, weirs, and tributaries. An average of 892,777 particles/km2 were discovered, with opaque spherules accounting for 45.2% of all microplastics. Fragments followed at 37.5%, transparent spherules at 13.2%, fibers at 2.5%, and miscellaneous types at 1.1%. Furthermore, Fendall et al. (2009) found that the spherules originate from plastic goods that were utilized as feedstock for plastics manufacturers, in addition to scrubbing granules and pellets found in air-blasting agents, industrial cleaners, cosmetic products, and many other products. Polystyrene accounted for 29.7% of the total polymer, with polypropylene coming in second at 16.9%, acrylate having different percentages of fewer complex units ranging from around five percent to more than five percent, polyester at roughly one to more than five percent, and PVC at less than two percent. Particle sizes range from 300 µm to 1000 µm, with a diameter of 78.6% less than the substrate.

86.4% of the particles analyzed were polymers that are produced in large quantities all over the world.

Suaria et al. (2016) examined the widespread presence of plastic marine debris in surface waters throughout the Mediterranean region by analyzing 74 neuston net samples with a mesh size of 200 µm. It was estimated that there are $3.1 \times 10^{12}$ microplastic particles in the waters of the Mediterranean, with a standard abundance of 1.65 particles/$m^2$. The remaining particles were fragments, with 93.3 percent being made up of pellets, foams, and films. Furthermore, the proportions of linked particles less than 300 µm, or about sand size, were 26% and 51%, respectively, and the mole scale entry was less than 500. FT-IR spectroscopy was used to identify 16 different polymer kinds, and it was discovered that polyethylene, or PE, was the most common type. two-dimensionally oriented on an increasing abundance scale, where yellow denotes less proportionate plastic mass and red, higher plastic mass.

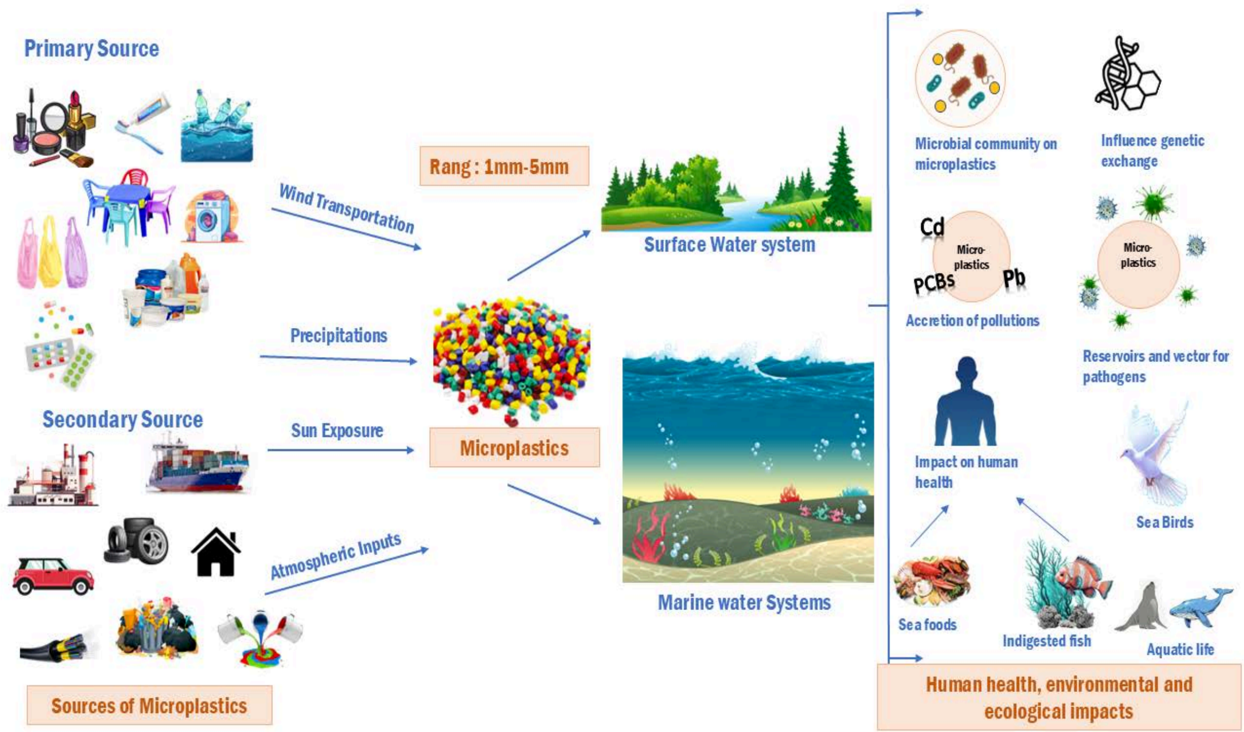
Alimba and Faggio (2019) reviewed articles, focused on oceans as sites of plastic accumulation and seabirds tested for the presence or consumption within their bodies at sea. Impeded nutrition leads to intensified malnourishment, skin scrapes and skeletal traumas. The ingestion of plastic blocks the digestive tract and it could damage your intestines, morbidity or even be fatal. However, the study presented that the imbalance of gene expression impairs crucial function for oxidative stress management and the nuclear factor E2-related factor (Nrf) signaling pathway in marine species  including vertebrates and invertebrates by microplastics. Microplastics cause oxidative stress, immune responses, genomic instability,and endocrine disruptions along with neurotoxicity,but also result in reproductive toxicity where embryotoxicity and transgenerational toxic effects are considered.

Ajith et al. in (2020) An essay on the distribution, availability, and impacts of microplastics on living things was studied. Nonetheless, this assessment addressed all of the previously listed topics and provided a briefing on the worldwide understanding shortcomings in each of the previously mentioned elements, emphasizing those gaps globally. Only 22.9%, or 44 out of the 192 nations in the globe, have microplastics reported to date, according to the research. The majority of these studies (38%), which focus on fish, have tragically disregarded other species, such as turtles, which is concerning since it might result in many more fatalities or significant injury to non-target animals. We also recommend that all aspects of microplastics study be continued, as many virgin marine environments and animals remain undiscovered. A microplastic limit level that corresponds to recognized risks as well as advised actions and techniques that are prepared for use in lowering plastic breakpoint values in marine ecosystems may be defined in a quantified regime.

J. Li et al. (2023) This study summarizes the current state of knowledge in this field by examining the potential effects of MPs precipitation on their physicochemical properties and marine life on the vertical mobility of MPs. The original distribution of MPs is based on their physicochemical characteristics. MPs' density, size, and form all influence their ability to settle in the water. Marine biota are mostly responsible for the transit of MPs into deep ocean waters by modifying the amount of and adsorption of MPs. Biofouling can alter the surface features of microplastic particles (MPs) and increase their overall density, hence influencing the downward movement of the plastic. Macroalgae have the ability to ensnare MPs particles by the generation of chemicals or by using electrostatic interactions. While benthic organisms on the ocean floor can move MPs from sandy grounds to deeper layers, MPs are consumed by swimming marine animals and expelled as fecal particles.To improve the downward movement of MPs, MPs can also be added to organic particles made by marine life, including sediments from the ocean or marine snow..

However, given the complexity of different maritime environments and MP properties, the testifying activities of MPs may be the result of several processes interacting. More research is needed to understand how the properties of MPs, marine life, and surrounding ecosystems impact MP accumulation in the marine realm in order to address this knowledge gap.

**Figure 07: A schematic diagram of microplastics introduction on living beings from the sources**



## 6.2 Microplastics Detection and Identification Methods

### 6.2.1 Raman Spectroscopy

The MPs in ten brands of single-use PET-bottled water from Thailand are quantified and qualified by Kankanige, U., & Babel, S. (2020) using Raman spectroscopy. The same approach was used to independently compare the MP concentrations in bottled water contained using two different packaging with glass. Individuals were sorted by 1) optical microscopy (≥50 µm) and 2) manually identified with Nile Red (> or =6.5 microns). The measurements were carried out with the use of confocal Raman spectroscopy (1–50 µm) and ATR-FT-IR (≥50 µm). The MP concentration was 52 ± 4 p/L in glass bottled water and 140 ± 19 p/L in single-use plastic bottled water. Plastic bottles had a much larger MP amount compared to this later. This fraction was 6.5 -- 20 µm and 20–50 µm MPs, with the latter two likely determining its difference in size from <1cm−2 (result not shown). Fibers comprised 62.8% of the particle count, followed by fragments. Optical microscopy imaging yields an average size of ≥50 µm particles 10 ± 1 p/L, not significantly different from the fluorescent-tagged Arabsame in that range (12 ± 1 p/L). These results imply that the influence of shear forces alone are likely to be similar in both techniques for MPs greater or equal 50 µm.

### 6.2.2. FT-IR Spectroscopy

Löder and colleagues responded to this need by presenting an assay that may serve as a standardized procedure for the study of small microplastics in environmental samples. (2015). There are insufficient methods to measure microplastics enriched on filters readily and quantitatively. Here, we take the first step in bridging this gap by investigating environmental samples for microplastics using focal-plane array detector based microscopy (focal plane-array

Fourier-transform infrared imaging). By iteratively optimizing our analytical approach (filter material, measuring mode, measurement parameters, identification protocol), we were able to measure the entire surface of filters with microplastics from marine plankton and sediment samples (>10 mm diameter) successfully. This was particle size appropriate measurements and could be done rapidly in comparison to chemical mapping using its high lateral resolution.

Hossain et al. conducted studies to assess whether IMF is applied under relatively less harmful conditions using stereomicroscopic/FTIR methods and FTIR imaging respectively, characterized MPs from water samples of ten branded and commonly used bottled waters. It also provided an estimate of per person oral consumption by MPs. Indeed, our results demonstrated the presence of MPs in all brands. The average number of MPs was 35 ±19 particles/L for all brands with a minimum and maximum value from 14±6.8 to56 ±23 particles/L, respectively. For most scientific purposes, we might assume that MP can be considered specific markers. Unfortunately, based on this standard, sires in the country under study had higher levels ofMP than other countries at any time compared respectively. They identified four different types of MPs — fibers, fragments, films and beads. Among these, fibers dominated (90%) the samples. MPs were categorized into 8 colors total with just over 94% of MPs being <0.5mm and a transparent color forming approximately ~66% of all particles counted Confirmed by FTIR (Powder).

### 6.2.3. Fluorescence Lifetime Imaging Microscopy (FLIM)

FLIM means Fluorescence Lifetime Imaging Microscopy. Monteleone et al. state that fluorescence lifetime imaging microscopy, a micro-spectroscopic technique to detect fluorescence lifetime of plastics, is a novel approach for plastic identification and characterization. We sought to find out if

FILM can be used in identifying and characterizing plastics. Six plastic types were subjected to exploration under FLIM. The plastics included ABS, PA6, PET, PLA, PPE, and PU. All six were plastic known as polyethylene except PA6. Both excitation wavelengths of 470 nm and 440nm were used with and without heat treatment. The results showed mean τ intensity weighted values of 3.519 (±0.090) ns for PET German Bottle, 3.564 (±0.126) ns for PET USA Bottle, 8.143 (± 0.060) ns for PPE, and 3.850 (±0.033)ns for ABS. Out of the 55 plastic comparisons, 52 were significantly differentiable using the mean intensity weighted τ and mean amplitude weighted τ values . Further, FILM offered the potential for phasor analysis, plastic can be characterized in the sub-micrometer range, and the visual ability for 3D-sectioning of samples, all which are likely to be important for the identification, as well as characterization of plastics in environmental and tissue samples .

### 6.2.4 Flow Cytometry

Tse et al., 2022 have quantified MPs in nine brands of bottled water using fluorescence microscopy of particles ≥50 μm and flow cytometry of particles <50 μm. The number of MPs in bottled water was between 8 and 50 particles per liter for particles ≥50 μm, and 1,570 to 17,817 particles for particles <50 μm. The abundance of particles contaminating mineral water was significantly higher than that of distilled and spring water.

### 6.2.5 Ultraviolet Light Photobox

Applying a similar method Welle and Löder (2019) described a novel way of identifying microplastic particles in bottled water by Hengstmann & Fischer. There are various methods to detect microplastics in samples of the environment. Visual identification is commonly adopted as it is easy to implement and cost effective in terms of procedure, but tends to yield biased results. Spectroscopic or chromatographic methods on the other hand are reliable but time-consuming and require expensive equipment. Nile red staining is an alternative and additional means to detect microplastics. The reliability and feasibility of Nile red staining and imaging in a UV light photobox was assessed within this study. The method was also compared to an alternative staining-based identification technique that uses a fluorescence microscope. The origin of the polymer fraction was determined by careful insight into μ-Raman spectra of identified microplastic particles.

# Chapter 07

## Materials and Methods

## 7.1 Introduction:

In Bangladesh, most of the local people in the metropolitan area use the water supplied by WASA. On the other hand, others fetch water from underground or use the surface waters like ponds, lakes and rivers. However, WASA uses the surface water like rivers, lakes etc. to distribute water to the customers. Similarly, packaged drinking water companies use these surface waters as their source of water. In the previous chapters, it was discussed how microplastics have polluted all the water sources. Marine water to riverine water, every water body is contaminated with microplastics for the excessive use and unplanned littering of plastic materials. Therefore, it can be predicted that microplastics can be present in our drinking water. Although, according to the companies, local packaged drinking water is refined several times, they should be free of microplastics and safe for drinking. Therefore, the purpose of the study is to find out if microplastics are present in local packaged drinking water. The methodology explains the processes to reach a result which will lead to the conclusion of the research to detect the presence of microplastics on the drinking water.

## 7.2 Aim of the study:

General Objective of the research:

To detect the microplastics in packaged drinking water.

Specific Objectives:

1. To find out microplastics in packaged drinking water using Nile Red dye.
2. To find out the number of microplastic particles on packaged drinking water bottles.
3. To find out the quality of drinking water sold on local markets.

4. To predict the number of microplastics consumed yearly by individuals through the packaged drinking water.
5. To find out possible ways to reduce microplastic consumption by drinking packaged water.

## 7.3 Apparatus and Reagents :

Apparatus:

1. Glass Beaker
2. Glass Conical Flask
3. Glass Pipette
4. Glass Petri Dishes
5. Bunsen Burner
6. Metal Tweezers
7. Filter Paper (0.22 µm pore size, 0.47 mm diameter)
8. Vacuum Manifold Filtration System
9. Rocker 400 Vacuum Pump
10. Light Microscope
11. Analytical Balance
12. Aluminum Foil
13. Glass Funnel
14. Pipette Pump
15. Clamp

Reagents:

1. Nile Red Powder (Sigma N3013-100MG, Technical grade)
2. DNase/RNase-Free Water (Solarbio Life Sciences)
3. Acetone
4. Distilled Water
5. Ethanol

## 7.4 Purpose of Using Some Reagents:

### 7.4.1 Sodium Dodecyl Sulfate (SDS)

SDS is a surfactant that is highly effective in case of removing oily stains from any surface. It has high solubility in water and can mix well with oil. At the same time, it is widely used in laboratories for purposes like lysing of nucleic acids, denaturing proteins and using it as an emulsifier or foaming agents. SDS is used also for observing microplastics because this surfactant reduces the surface tension and thus it can effectively extract the microplastics. To clarify, SDS habitually can digest the organic materials of the samples and thus it can detach the microplastics from the organic materials. As a result, microplastic particles are more visible under the microscopes. Also, SDS surfactant is compatible with analytical techniques like Fourier-transform infrared spectroscopy and Raman spectroscopy, the two preferable techniques to characterize microplastics (Randhawa, 2023).

### 7.4.2 Ethylenediaminetetraacetic Acid (EDTA):

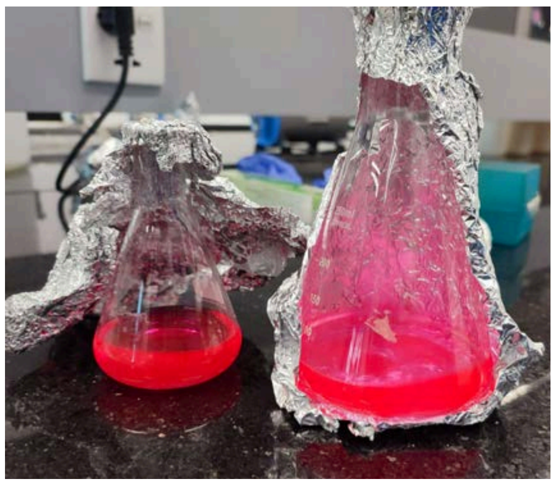
EDTA is extensively known as a chelating agent that can remove free metallic ions, including heavy metals from solutions (George & Brady, 2024), and this EDTA is also a useful reagent in the investigation of microplastics on water. This reagent is mostly used on the environmental samples to remove the toxic heavy metals from the samples.

### 7.4.3 Nile Red:

Microplastics particles can be more visible if they are stained with a dye, especially if stained with any fluorescence dye. Among many fluorescence dyes, namely oil red EGN, Eosin B, Rose Bengal, Nile Red, Crystal violet, Safranin-T, Trypan Blue, Rhodamine B (Shruti et al., 2022), Nile Red is the most potential and widely used fluorescence dye because Nile red can stain a wide variety of microplastic particles like polyethylene (PE), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), polyethylene terephthalate (PET) (Bianco et al., 2023) (Gao et al., 2022)

Nile red dye is hydrophobic and at the same time lipophilic. It explains that nile red can easily dissolve in lipids such as fats and oils and binds to hydrophobic materials. Moreover, this maroon colored powder is sensitive to light, air and moisture, hence it requires handling properly. Most of the plastics, namely, PE, PP, PS etc are made of hydrophobic polymers and they are lipophilic as well. This characteristic allows microplastics to bind to nile red dye and then the bound microplastics-dye exhibits strong fluorescence under a specific wavelength. Nile red has high sensitivity hence smaller particles are easily detected by this dye. This dye can be prepared by dissolving it in methanol (Erni-Cassola et al., 2017), ethanol, or acetone (Cole, 2016) and chloroform (Tamminga et al., 2017) shown in figure 08. The function of different solvents are similar, so they are used based on their availability. In laboratories, chloroform and methanol are not selected often because of their toxicity. On the other hand, ethanol and acetone both individually work similarly to dissolve the dye, however, research found that mixing acetone and ethanol in equal proportion while dissolving the dye results in a better stain on the samples (Konde et al., 2020).

**Figure 08: 10 µg/ml and 20 µg/ml NR solution**



Moreover, according to Shim et al., (2016), after dissolving the dye in acetone, diluting the solution on n-hexane enhances the staining intensity. Staining efficiency also relies on the concentration of the dye. There are several concentrations of nile red that stains the sample at different temperatures for different durations. For example, 5 µg/ml, 10 µg/ml, 20 µg/ml and 500 µg/ml are the most applied concentrations of nile red dye. These different concentrations require different incubation durations and temperatures. According to Shim et al., (2016), samples need to be stained by 5 µg /ml dye and then incubated for 10 minutes at room temperature and then washed with n-hexane. Maes et al., (2017) suggested using 10 µg /ml dye on the samples and incubating the samples on a shaker at 100 rpm for 30 minutes.  Cole (2016) also recommended incubating the samples for 10 minutes after a short vortex. Hence, the effective temperature for staining the samples with nile red dye is 200C, 40 °C, 50 °C, 60 °C and staining time ranges from 5 minutes to 60 minutes (Konde et al., 2020) and extends to 5 hours.

### 7.4.4 DNase/RNase-Free Water:

DNase/RNase-free water is a purified water that is free from any kind of impurities and nucleases like DNase (deoxyribonucleases) and RNase (ribonucleases). To make it pure, the water is deionized, filtered and autoclaved. This sterile water is mostly used for molecular diagnostics but is also an useful alternative of ultrapure water for microplastics identification experiments. Since microplastics are ubiquitous, it is necessary to maintain a very controlled area to minimize the exposure of plastics. Along with using glass apparatus, it is also necessary to wash them to get rid of any kind of microplastics. Therefore, the glasswares need to be, at first, washed with tap water, then with distilled water and then with the DNase/RNase-Free Water shown in figure 09. Then they must be covered immediately with a clean aluminum foil to prevent any contamination. Moreover, this water can also be used as the negative control and environmental blank sample.

**Figure 09: DDNase/RNase-Free Water (Blanks)**

## 7.5 Purpose of the Processes:

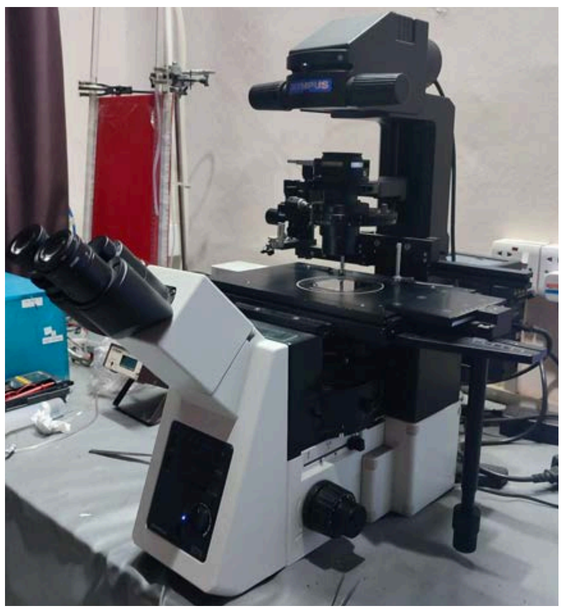
### 7.5.1 Filtration:

To isolate microplastics from any other particles, filtration is the most important process. Among different forms of filtration, for instance, membrane filtration including microfiltration, ultrafiltration, electrochemical filtration like electric field gradients (Thompson et al., 2021), to filter out the microplastics, microfiltration is the best suitable for university laboratories. In the membrane filtration process, pore size ranges from 0.1µm to 1.0 µm. Pore size 0.22 µm and 0.45 µm are frequently used filter-papers for microplastics isolation. The water can be filtered out in two ways, one is a traditional filtration method using glasswares and the other is using a vacuum manifold. In both of these methods, a vacuum pump is used for the suction of the water samples. On the glassware filter, the filter paper was placed in between the flask bottle and the funnel. Both are held by an aluminum clamp. On top of the flask bottle, there is a filter head for holding the filter paper and a sidearm to evacuate the air by the vacuum pumps.in contrast, the vacuum manifold is made of stainless steel. There are three to six branches that do the filtration. The funnels are also made of stainless steel that can hold 100ml to 500ml liquids. Each of the branches are controlled by lock knobs. The waste bottle collects the water after filtration. Here, also a vacuum pump does the filtration and takes the wastewater to the waste bottle by a tube.

### 7.5.2 Fluorescence Microscopy:

Fluorescence microscopes are also optical microscopes that use fluorescence unlike bright-field microscopes. When the sample, labeled with fluorescent dye, is observed under the fluorescence microscope, a light of selected wavelength emits from the light source and falls onto an excitation filter. Then the light excites the sample and that results in emission of high wavelength (Karki, 2024). Depending on the specific wavelength, the emission filter shows the color. For most of the samples, the excitation wavelength is selected from a range that will emit the light in either green or in blue color. Confocal fluorescence microscopy, infrared microscopy, inverted microscopy are different kinds of fluorescence microscopy shown in figure 10. Similarly, phase contrast microscopy can also be used to detect microplastics, although this is not the best alternative of other fluorescence microscopes.

**Figure 10: Fluorescence microscope (olympus ix73)**



## 7.6 Method

### 7.6.1. Step 01: Sample Collection and Washing:

A total of thirty six 500ml drinking water bottles were purchased from local markets, located in Mohakhali, Merul Badda, Gulshan 2 and Banashree (Table 2). The bottles were purchased in pairs but from different markets of different locations, so that their batch number and manufacturing date could not match. Three sets of 10 local brands and 2 international branded drinking water bottles were purchased. Each of the sample bottles were at first rinsed with tap water to wash off the surface dust from it. Then they were washed twice with the distilled water, then kept them inside the laminar hood.

**Figure 11: Different packaged water bottles as samples purchased from different stores.**

**Table 02. Samples Names, Volume, Purchase Location, Manufacturing Batch and Date**

| *Brand No.* | *Water Bottle Brands* | *Volume* | *Quantities* | *Location* | *Manufactured Batch No.* | *Type of Plastics Bottle* | *Manufacturing Date* |
|---|---|---|---|---|---|---|---|
| ***Brand 01.*** | *Jibon* | *500ml* | *3* | *Mohakhali* | *Batch-122[BE]* | *PET* | *1/5/2024* |
| ***Brand 02.*** | *MUM* | *500ml* | *3* | *Merul Badda* | *Batch-K2* | *PET* | *19/4/2024* |
| ***Brand 03.*** | *Fresh* | *500ml* | *3* | *Mohakhali* | *Batch-159(AF1)* | *PET* | *6/5/2024* |
| ***Brand 04.*** | *Deshbandhu* | *500ml* | *3* | *Mohakhali* | *Batch-068. 228* | *PET* | *3/5/2024* |
| ***Brand 05.*** | *Sun* | *500ml* | *3* | *Gulshan 2* | *Batch-L-2.I28* | *PET* | *7/5/2024* |
| ***Brand 06.*** | *Aquafina* | *500ml* | *3* | *Merul Badda* | *Batch-026* | *PET* | *22/4/2024* |
| ***Brand 07.*** | *Kinley* | *500ml* | *3* | *Banasree* | *Batch-105 10:13* | *PET* | *8/5/2024* |
| ***Brand 08.*** | *Pusti* | *500ml* | *3* | *Banasree* | *Batch-068. 228* | *PET* | *2/5/2024* |
| ***Brand 09.*** | *Acme* | *500ml* | *3* | *Merul Badda* | *Batch-099 10:21:21* | *PET* | *4/5/2024* |
| ***Brand 10.*** | *Pran* | *500ml* | *3* | *Banasree* | *Batch-5/7* | *PET* | *7/5/2024* |
| ***Brand 11.*** | *Sparkling Water Perrier* | *700ml* | *3* | *Gulshan 2* | *Batch-3179730011857* | *Green Colored Glass* | *15/2/2024* |
| ***Brand 12.*** | *Evian* | *500ml* | *3* | *Gulshan 2* | *Batch-30683200 84602* | *PET* | *25/04/2024* |

### 7.6.2. Step 02: Surface Cleaning:

Beforehand, the laminar hood was wiped with 10% bleach and then with 70% ethanol. The air circulation of the room was controlled and only a limited staff access was maintained. Before handling the samples, lab coats were worn, hair was covered with hairnet, face was covered with face masks and hand gloves and shoe covers were also worn to prevent contamination.

### 7.6.3 Apparatus Cleaning:

To minimize the contact of plastics during the experiment, all the apparatus chosen for the experiment was made of glasses. Glassware includes conical flasks, glass beakers, glass pipettes, glass petri dishes, flask bottle, filter head and funnel for filtration. Each of the glassware was washed with dishwashing detergent by tap water. It went through a second wash with the distilled water and then with the DNase/RNase-Free Water for maximus cleaning. Each of the glass containers were sealed with aluminum foil to prevent air exposure.

### 7.6.4. Step 03 : Filtering of Distilled Water:

Except the sample water, two other types of water were used in the experiment and these water need to be free from microplastics as well. The distilled water used in the experiment was made in the university laboratory and any form of contamination could be presented there. So all the distilled water used for the experiment was filtered before using. Since the glass membrane filter was used in this process, the flask bottle, funnel, filter head were washed beforehand with pure

DNase/RNase-Free water. Filter paper that had the pore size 0.22 µm and 47 mm diameter was also used here to filter the water. The filtered water stored on the flask bottles were then transferred to a clean conical flask. These water were filtered in required amounts and repeated whenever necessary.

### 7.6.5. Step 05: Reagent Preparation:

Glass apparatus were used for preparing and storing the reagents to minimize the contamination. According to Konde, (2020), the staining capacity of plastic particles depends on the concentration of nile red. Therefore, nile red dye was prepared in two different concentrations. The first staining solution was prepared by dissolving 1 mg Nile Red on 100 ml acetone to get a final concentration of 10 µg/ml. Another solution had the final concentration of 20µg/ml. Reagent acetone was drawn up from the glass bottle to a clean conical flask using a 50 ml glass pipette. The flask was covered with a piece of Al foil. Next, 1 mg and 2 mg of NR powder was weighed on the analytical balance correspondingly. These powders were directly added to the acetone and when they were dissolved completely, they were subjected to the test. Also, the dye solution needs to be covered in Al foil completely as NR is photosensitive. The residual dye was stored in the dark at -4℃.

### 7.6.6. Step 06 : Positive and Negative Controls:

From the samples bottles, some PET plastics were shaved from the bottles using a scissor. These plastic shavings were then turned into smaller pieces using sand papers. These were made to use as the positive controls. On two clean conical flasks, these pieces were added and covered it with foil to proceed in further research. On the other hand, 500 ml of DNase/RNase-Free water was taken on two conical flasks and they were covered in foil as well. These bottles were used as negative controls.

### 7.6.7 Step 07: Setting Environmental Exposure:

On two clean conical flasks, 500 ml DNase/RNase-Free water was kept inside the laminar hood, where the reagents were prepared and samples were stained. These blanks were maintained to measure the environmental exposure of microplastics particle contaminations.

### 7.6.8. Step 08: Staining the Samples:

Each of the sample bottles were stored inside the laminar hood. These bottles were carefully opened there so that plastic particles of the bottle cap can not contaminate the sample. From three sets of bottles, one set was kept aside for direct filtration. From the other two sets, on one pair of bottles, 5 ml dye from 10 μg/ml stock solution was transferred inside the sample bottles. On another pair, 5 ml dye from 20 μg/ml stock solution was dispensed inside the sample bottles. For both of the concentrations added on the samples, a 10 ml glass pipette was used. Following that, the samples were covered with Al foil and shaken vigorously to give them a proper mix. The samples were then incubated on the shaker for 5 hours at 37.5℃. This temperature allows the plastic particles to attach efficiently with the nile red. Two positive controls, two negative controls and two environment exposure controls were indistinguishably stained with two concentrations of NR dye and incubated in the shaker like the samples.

### 7.6.9. Step 09: Filtration of the Samples:

After incubation all the samples and controls were subjected to the filtration process. Prior to that, the membrane filter machine was equipped properly. All the windows and doors were locked to prevent unwanted contamination. The filter cups were washed with distilled water and rinsed with DNase/RNase-Free water. The lid was off until the further process began. A bunsen burner was lit beside the filter. A tweezer was heated there and a filter paper was placed on the filter membrane using the tweezer. The vacuum pump was turned on and the samples were poured on the filter cups after adjusting the knob. Each of the stained samples were filtered in this way and the filter papers were placed on the clean glass petri dishes. The filter papers were transferred to the petri dish by a sterile tweezer. The petri dishes were then kept inside the laminar hood to dry out the papers. Similarly, the unstained sample bottles were filtered on the vacuum filter.

### 7.6.10. Step 10: Observation on Optical Microscope

The unstained sample set was observed under the light microscope. The filter paper was divided in four quadrants to examine the whole paper properly. Using Inverted Nikon Microscope on 40X magnification, some long microfibers were observed on most of the samples. The list of particles counts are mentioned in graph 01.

### 7.6.11. Step 11: Observation on Fluorescence Microscope:

The samples were observed under the fluorescent microscope. For this research, an Olympus IX73 microscope was used, with the filter combination of 450 nm blue excitation and 550 nm green emission. These wavelengths allowed the NR dye to emit the sample and observe it under the microscope. The lenses were set in 10x magnification. On the monitor, the shapes of the samples were seen in monochrome; the bar was set to 50 µm. For each of the samples, the filter paper was divided into four parts and on each quadrant, the particle numbers were counted. In this way, the total particle number in a sample was counted and listed on a table mentioned below. Also the pictures of the samples were used for measuring the size of the particles using the ImageJ software.

**Workflow:** The Brief Description of Microplastics Identification Methods and Observation

**Figure 12: The brief workflow of microplastics observation on samples**

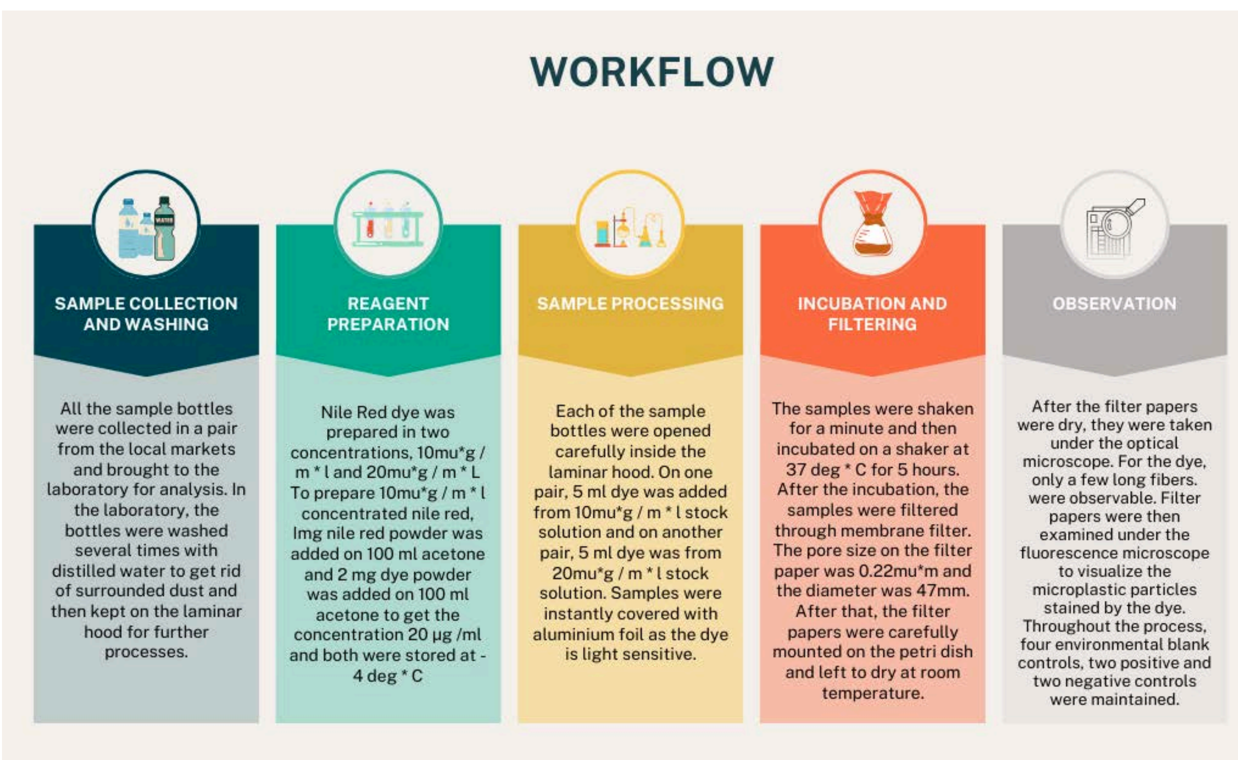
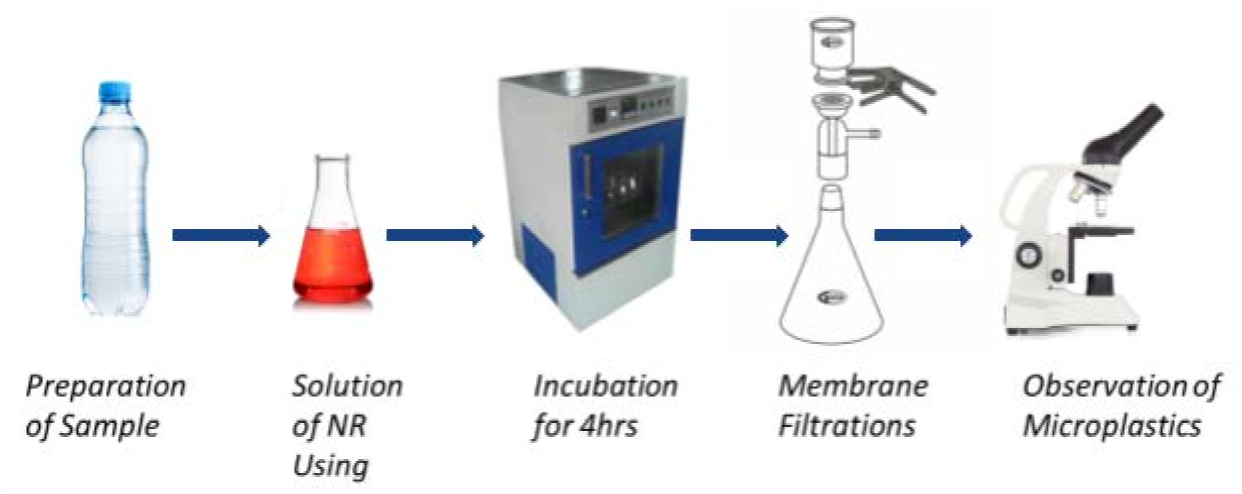


**Figure 13: The steps of conducting microplastics filtration process**




## 7.7 Survey Methodology

A cross-sectional survey is an observational study that measures the exposure and the outcome at the same time in a sample of a population. Dhaka city population based digital survey instrument for medical thesis supervisors assessment. Aspects of the questionnaire measured by concurrent validity, predictive validity, sensitivity to change, discriminant validity and mean scores for different populations were summarized. The survey was conducted inside Dhaka city. The questionnaire included personal information such as gender, age and weight of the respondents. The data about their daily intake of water and consumption of water using local packaged plastic bottles, their most preferred local water bottle brand, reuse of the plastic bottles and purification method of drinking water were also collected. The data were presented in charts and graphs which were then correlated with other information.

## 7.8 Ethical Statement

The participants had voluntarily participated in the survey and gave consent to take part in the survey and use their data on the investigation. They were informed regarding the study's objective and methodology.

## 7.9 Research Domain and Disciplines

This investigation was completed in-person over email, groups, and other online spaces. The survey was conducted between 19 Aug, 2024 and 19 Oct, 2024 As such, before any data collection has been carried out an online form must be presented to collect the appropriate consent. The study covered 580 respondents. Most of the survey respondents were emailed the form. Patients' ages were between 15 and more than 65. The participants were from a variety of occupational backgrounds and both genders and respondents lived only in Dhaka.

## 7.10 Questionnaire

**Public Health Survey: Understanding Hydration Habits**

You are invited to participate in a survey about drinking water habits. The purpose of this survey is to gather information about daily drinking water habits using plastic bottles based on their age, weight and employment status. Your participation is entirely voluntary, and you may withdraw at any time without any consequences. This survey will take approximately 5 minutes to complete. No personally identifiable information will be collected and the response will be solely used for the research purpose.

**Consent Statement:**

By proceeding with this survey, you are agreeing to participate in this study. If you have any question about the survey or the research, please use the comments section at the end of the form. Thank you for your time and participation.

- ☐ I agree to participate

**Where are you from?**

- ☐ Inside Dhaka

**Your Age**

- ☐ 16-25
- ☐ 26-35
- ☐ 36-45
- ☐ 46-55
- ☐ 56-65
- ☐ 65 and above

**Your Gender**

- ☐ Male
- ☐ Female

**Select Your Employment Status**

- ☐ Student
- ☐ Employed
- ☐ Unemployed
- ☐ Freelancer
- ☐ Other: ___________________

**Your Weight (answer in number)**

________________ kg

**Daily Water Intake in liters:**

- ☐ 1-2 L
- ☐ 2-3 L
- ☐ 3- 4 L
- ☐ 4-5 L

**Water Intake Using Plastic Bottles, in ml (Recent Purchase)**

- ☐ 500 ml
- ☐ 1000 ml (1L)
- ☐ 2000 ml (2L)
- ☐ 5000 ml (5L)
- ☐ 0 L

**Do You Reuse Your Purchased Plastic Bottles For Storing Drinking Water?**

- ☐ Yes
- ☐ No
- ☐ Often

**Which of The Following Do You Prefer Most For Drinking Water?**

- ☐ Direct Tap Water
- ☐ Filtered Water
- ☐ Boiled Water
- ☐ Purchase Water Bottles
- ☐ Raw Water

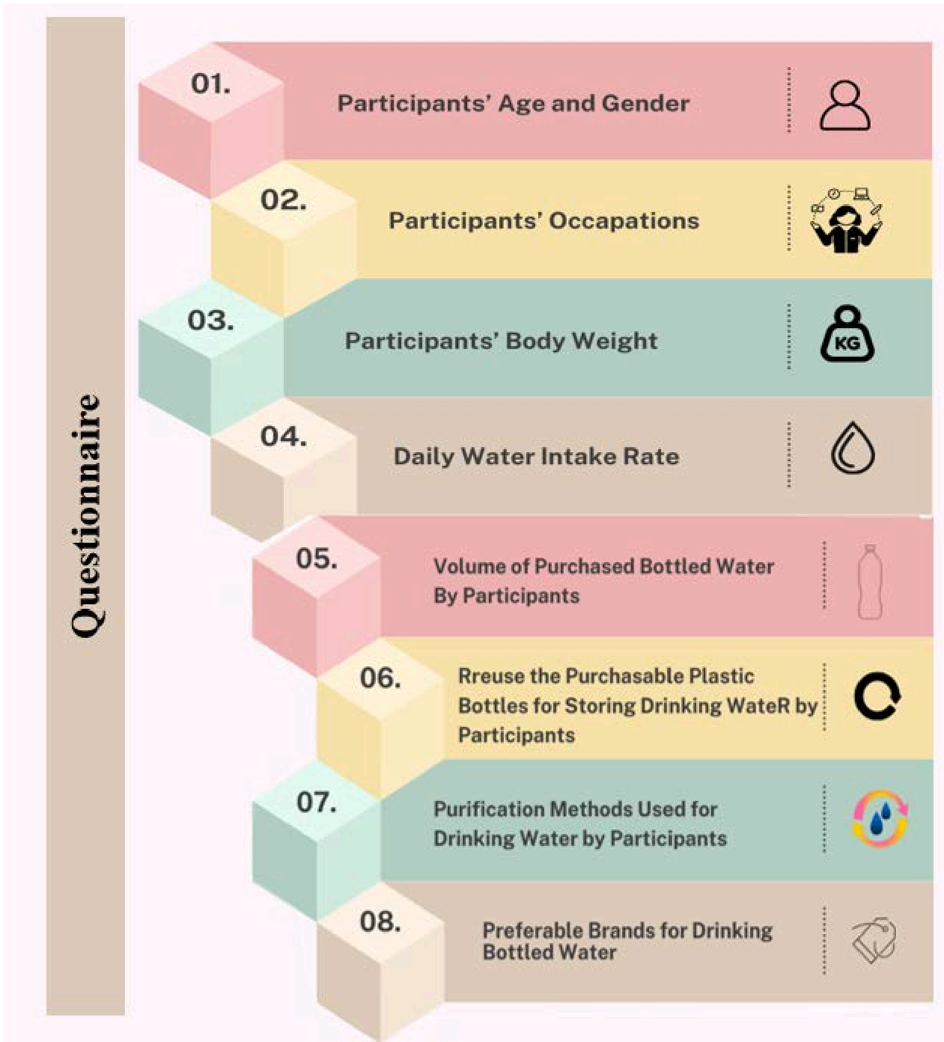
**Which Water Brand Do You Prefer To Drink Most (You can select more than One)**

- ☐ Mum
- ☐ Aquafina
- ☐ Fresh
- ☐ Kinley
- ☐ Jibon
- ☐ Acme
- ☐ Sun
- ☐ Pusti

☐ Deshbandhu

☐ Pran

**Figure 14 Flowchart of Questionnaire**




## 7.11 Statistical Survey and Data Analysis

All the data from sample observation and survey were analyzed using R programming and R studio. Mean, standard deviation, percentage of variables, regression analysis of microplastic particles on three sets of samples, correlation of microplastics particles on different sample bottles were calculated.

# Chapter 08

# Result

## 8.1 Introduction

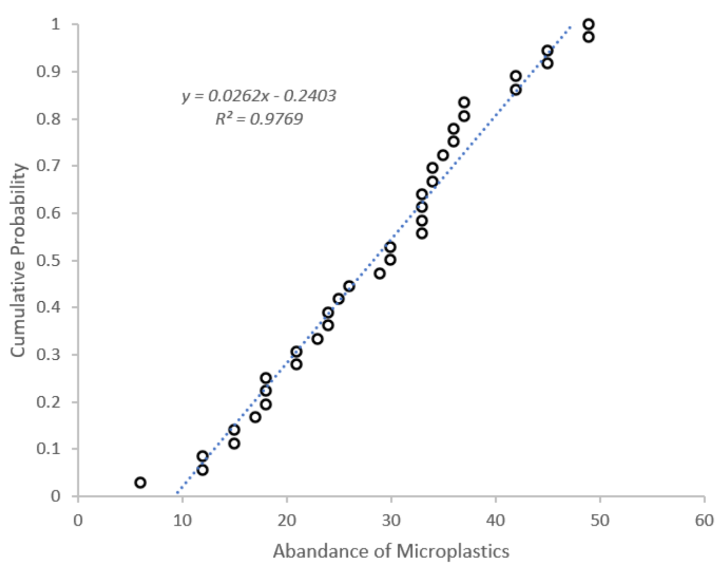
This chapter contains the research finding of microplastic pollution in packaged water bottles through microscopic analysis. This study impacts these mp pollution on drinking water specially plastic water bottles through fluorescence microscopic analysis as well as better understanding the presence of MP particles in packaged drinking water bottles in dhaka city. The overall proper methodology was carried out in the study introduced in the Chapter 7, included collection of samples, experimental setup and microplastic identifications and characterization. This chapter represented the detailed results of the laboratory experiments which carried out the quantification and identification of microplastics from the determined brands. In this study, the characterization of the samples were determined according to different sizes, distribution , shapes and morphology of microplastics. The concentration of microplastic particles was measured by each filtration. The sizes of the microplastics were measured through image J software and the statistical analysis performed to calculate the abundance of Microplastics as the brands are repeated (using same batch PB Brands) per filter. To express the abundance of microplastics calculated the mean $\pm$ SD. The structure of microplastics particles were distinguished into major three groups, including microbeads, fibers and fragments whereas the presence of films, foams, and other shapes are very limited. The microbeads are sphere edged and irregular in shapes with the less than millimeter size ranges (Isobe, 2016) , additionally, fragments are rigid and sharply edged but their presence is irregularly shaped. Fibers are thin long chains, flat mostly transparent and sometimes presence in color is identified (Viršek et al., 2016) . During observation, fragments found in Fluorescence microscopy analysis after using NR dye in samples but none of them were observed under Optical microscope. However, fibers are mostly transparent and sometimes have a blue color identified in optical microscopy as shown in figure 17. SDS was used to clean surfaces and samples, the

DNase/RNase free water was used as blanks which have the same work method like Ultrapure water after filtration process. Therefore, the MP particles in blanks are null due to preparing the blanks with filtered water that was prepared through the method of membrane filtration process.

### 8.2.1 Occurrence and abundance of microplastics in packaged drinking water bottles

Microplastics identified in packaged water bottles are raising concern for water intended for human consumption. Total 12 different brands with fewer of the same origin are collected from different general stores, super shops and shopping malls in Dhaka city. The laboratory analysis is done in three batches with two different concentrations of Nile Red dye dissolved in acetone. The microplastics particles are found in (N= 36) samples from these brands as shown in Table 02 and the quantification analysis of these brands are shown in graph 01.

**Graph 01 : Distribution of Microplastics Abundance Cumulative Probability in Different Brands of Packaged Drinking Water bottles and The Particles Counts**



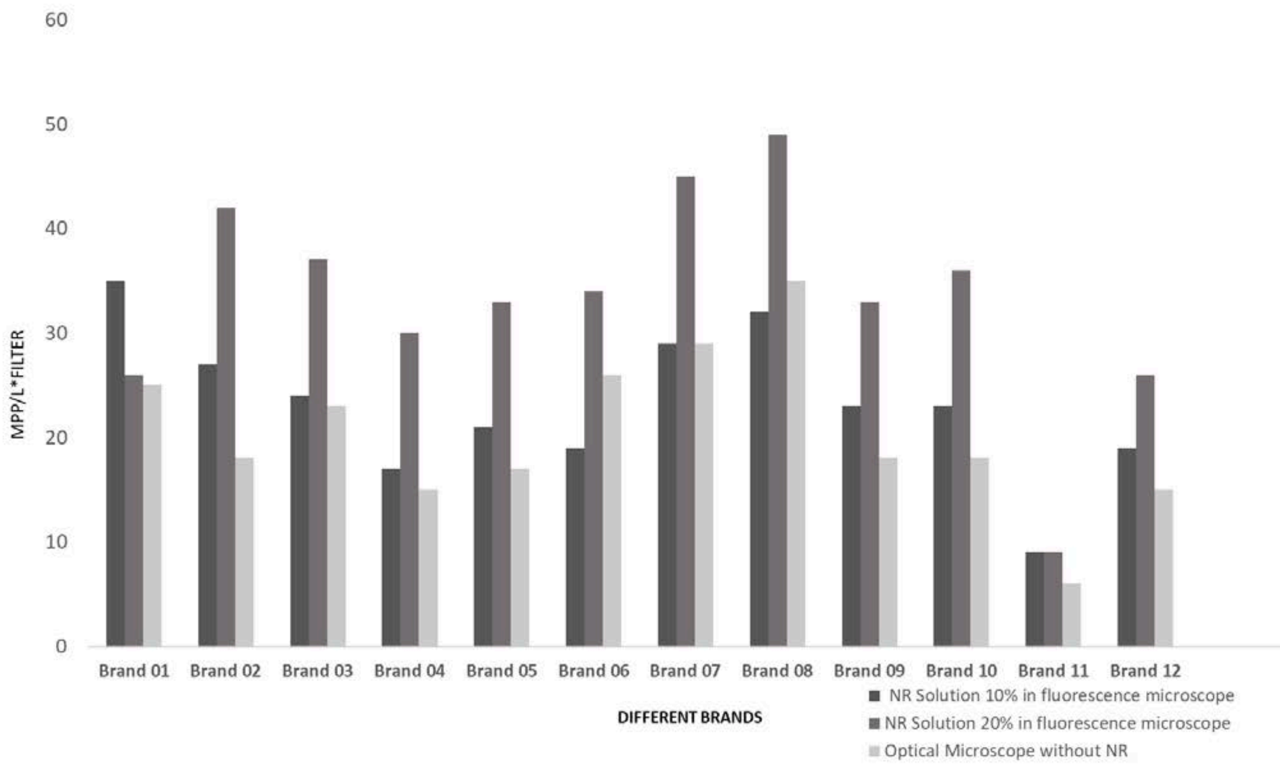
Graph 01 represented the cumulative probability distribution and microplastic concentration in plastic drinking bottle water. The statistical illustration of distribution of microplastics with specific sets of samples provided the information on the range of concentration and to locate particles at different levels. The model showed high accuracy, as the R-Squared Value 0.9763. the value signified that the cumulative probability distribution model explained that the variation observed in microplastic concentration in bottle water was approximately 97.63%

### 8.2.2 Microplastics Counts from Different Applied Approaches

Analysis of quantification showed that the three specific brands with different methods to quantify the MP particles. The bar chart illustrated the MPP from different brands analyzing the data with dye and out dye respectively in fluorescence microscope and optical microscope. Overall the utmost of MP particles has shown in Brand 08 which has the highest mean and SD (38.67 ±9.07) of items/filter whereas the least particles has shown in Brand 11 which has the lowest mean (8± 1.73) of items/filter.

Furthermore, the second highest mean value is shown in Brands 07 and 02 respectively (34.33 ± 9.23) items/filter and (25.67±7.1) items/filter. Therefore, comprehensively, amount of MPP on average using NR dye in acetone solution is 10% per sample is (23.17±7.04) items/filter and the MPP on the average using NR dye in acetone solution is 20% per sample is (33.33±10.4) items/filter. The MPP counts have found a major difference between two concentrations of dye using whereas, the quantifying of MPP counts has dramatically changed, found in two different approaches. Such as, the average MPP in optical Microscope is (20.42 ±7.63) items/filter which is comparatively less amount of MPP than fluorescence microscopic analysis.

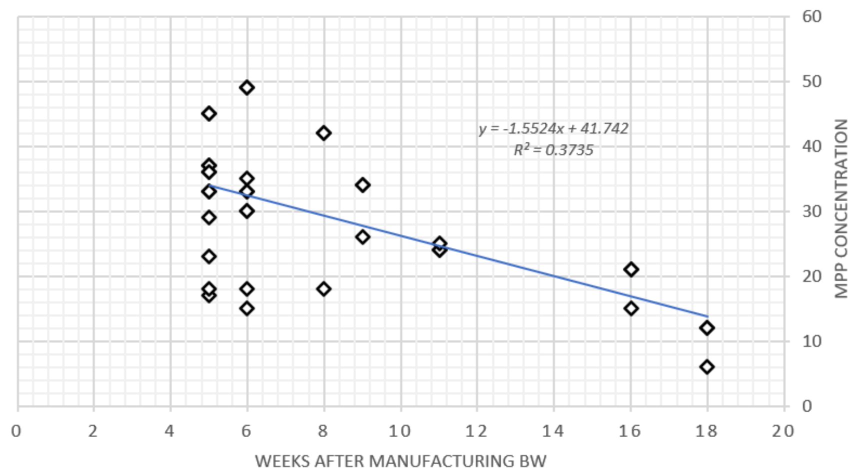
**Graph 02: The Abundance of Microplastics in Different Brands of Packaged Water Bottles**




### 8.2.3: The Abundance of Microplastics in Different Weeks After Manufacturing

There was a significant difference found between microplastic quantification as the manufacturing dates of our samples were different. The time period between the manufacturing dates of samples and our experiment conducting varies the microplastics particle and the maximum time preserved in the water bottles was Brand 11, Brand 12 and Brand 06 for 18 weeks, 16 weeks, 9 weeks respectively. Two brands 11 and 12 were international water bottles made of glass and plastic respectively. Brand 1 and Brand 2 were preserved for 11 and 8 weeks simultaneously. Other brands were preserved for a maximum of 5 to 6 weeks.

**Graph 03: The Abundance of Microplastics in Different Weeks**




Graph 03, illustrated that the increase of microplastics varies with decreasing time of preserving water in plastic bottles. Apart from specific brands most of the microplastics concentrated in 5 and 6 weeks after manufacture. The plot showed the weak influence as the R-squared value is 0.3735 the value signified that the distribution model explained that the variation observed in microplastic concentration in bottle water was approximately 37.35%.

## 8.3 ANOVA One Way Test to check the association between two different approaches : Fluorescence microscopy and Optical Microscopy

**Null hypothesis** : There is no correlation between the Microplastics particles detected in fluorescence microscopy analysis using NR solution and optical microscopy analysis without using NR solution in samples.

**Alternative hypothesis** : There is a correlation between the Microplastics particles detected in fluorescence microscopy analysis using NR solution and optical microscopy analysis without using NR solution in samples.

Here, the purpose of the ANOVA test is to test these hypotheses where the p-value determines whether the null hypothesis is rejected or not. If the p-value is less than the significant level that is 0.05 then we reject the null hypothesis in favor of the alternative hypothesis.

**Table 03. Results from ANOVA One-Way test for difference in the two approaches between fluorescence microscopy and optical microscopy.**

***Anova: Single Factor***

*SUMMARY*

| *Groups* | *Count* | *Sum* | *Average* | *Variance* |
|---|---|---|---|---|
| *NR Solution 10% in fluorescence microscope* | *12* | *278* | *23.1666667* | *49.6060606* |
| *NR Solution 20% in fluorescence microscope* | *12* | *400* | *33.3333333* | *108.060606* |
| *Optical Microscope without NR* | *12* | *245* | *20.4166667* | *58.2651515* |

***Anova: Single Factor***

*ANOVA*

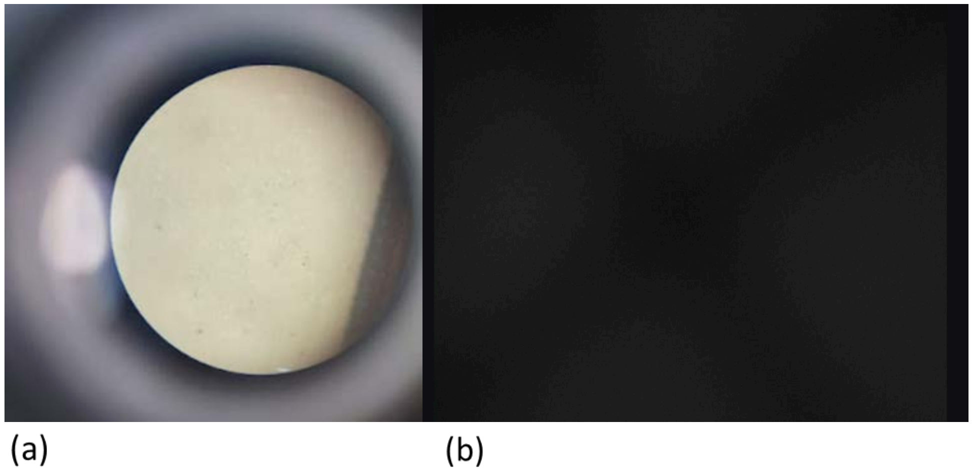
| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
|---|---|---|---|---|---|---|
| *Between Groups* | *1111.05556* | *2* | *555.527778* | *7.71809985* | *0.00177899* | *3.28491765* |
| *Within Groups* | *2375.25* | *33* | *71.9772727* | | | |
| | | | | | | |
| *Total* | *3486.30556* | *35* | | | | |

In table 03, the P-value is 0.00177899. Since this is less than the significant level α which is <<0.05 and the F crit is 3.28491765 , we can conclude that there is a significant difference between the means of the two different approaches.

## 8.4 Analysis of Blanks and Environment Exposure

In chapter three describe the methodology of blank and environment exposure. The quantity of Microplastics items and particles is null(0) due to the double filtration process applied as shown in figure 15. The laboratory apparatus and equipment are thoroughly clean with the double filter Distilled water and DNase/RNase free water. DNase/RNase free water is used as an alternative to Ultra Pure Water. Nevertheless, crushed plastics particles from selected samples are used as positive control. Although strictly maintained the cleanliness to avoid the external contamination, the background contamination result is also measured to quantify the microplastic particles approximately. However, the environment exposed to microplastics is measured ≤5 (less than 5) particles that are minimized from each sample.

**Figure 15. Blank under different microscopes. (a) Indicated optical microscopic view of blank and (b) Indicated fluorescence microscopic view of blank.**



## 8.5 Microscopic Analysis of Microplastics in Size Variation

### 8.5.1 Abundance of Large Microplastics (≥1mm)

Large microscopic particles, sizes are greater than 1mm in packaged drinking water bottled samples are stained with 10 µg/ml stock solution and 20 µg/ml stock solution of Nile Red solution respectively samples and quantified by fluorescence microscope with 450 nm blue LED laser light (450 nm) and green LED laser light (550 nm) emission filters. The particles are counted after subtracting the background contamination. According to table 04, microplastics particles that are greater than 1mm were detected from all drinking water bottled samples stained with 10 µg/ml nd stained with 20 µg/ml stock NR solution, where the individually average microplastics abundance of bottled water samples were considering 2 size categories : >1 to 3 mm and >3 to 5 mm. The particle concentration total across all brands found while using 10 µg/ml NR solution were 35 particles per litter and per filter for >1 to 3 mm and for >3 to 5 mm 38 particles per litter and per filter, whereas, the particle concentration total across all brands found while using 20 µg/ml NR solution were 58 particles per litter and per filter for >1 to 3 mm and for >3 to 5 mm 69 particles per litter and per filter. Individually, the MPP ranging for both the size ranges found 1 to 8 particles in each sample and there is a significant difference in MP particle concentration ($p<0.05$) in these two size ranges.

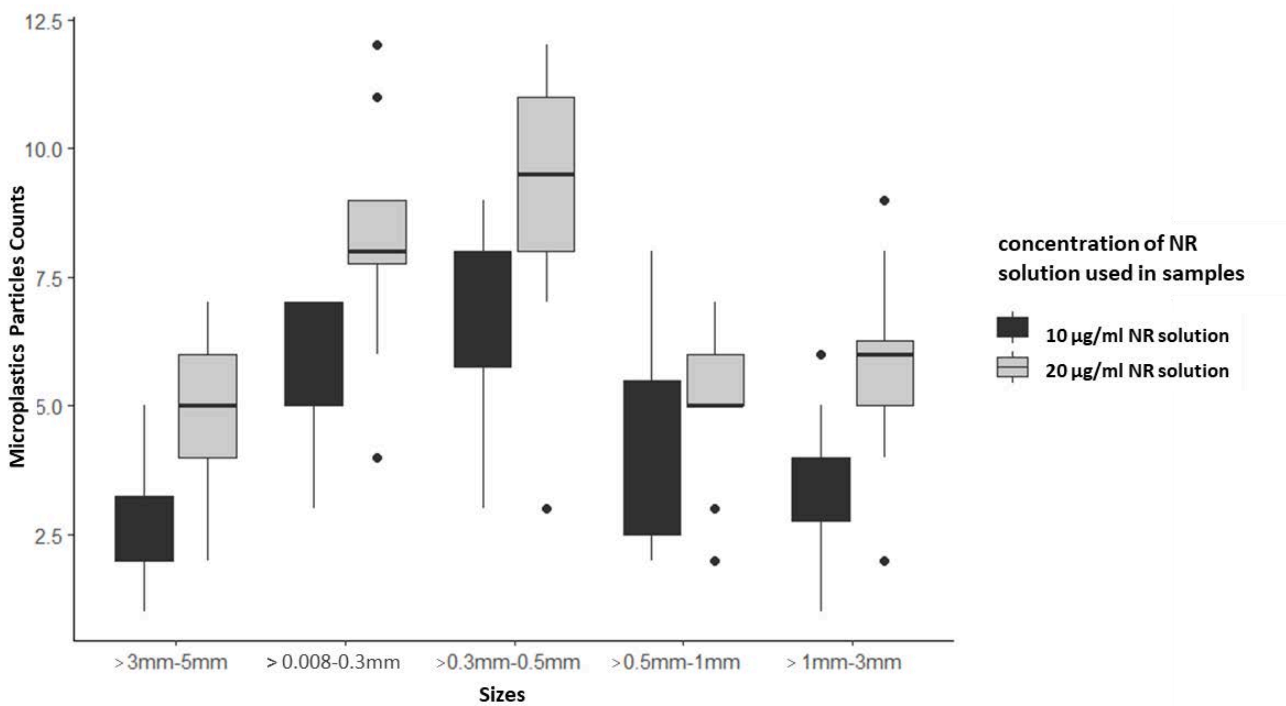
**Table 04. Microplastics Quantification with Different Sizes Using Different Concentrations of NR Solutions.**

| | ***Size Fractions*** | | | | |
|---|---|---|---|---|---|
| | ***>3mm-5mm*** | ***>1mm-3mm*** | ***>0.5mm-1mm*** | ***>0.3mm-0.5mm*** | ***>0.08mm-0.3mm*** |
| ***Using 10 µg/ml NR Sol*** | *35* | *38* | *53* | *81* | *71* |
| ***Using 20 µg/ml NR Sol*** | *58* | *69* | *62* | *99* | *111* |

### 8.5.2 Abundance of Small Microplastics (≤1mm)

To quantify microplastics which are less than 1mm in the individual samples, drinking bottled water after NR staining were analyzed by the fluorescence microscopy approach. As shown in table 04. smaller MPP detected according to three size categories: >0.5mm-1mm, >0.3mm-0.5mm, and >0.08mm-0.3mm. the mp particle concentration total across all brands found while using using 10 µg/ml NR and 20 µg/ml NR solution were 53 and 62 respectively per filter for >0.5mm-1mm, additionally, for >0.3mm-0.5mm mp particles detected 81 and 99 and for >0.08mm-0.3mm mp particle detected 71 and 111 respectively. Individually, the particles of microplastics ranging for the size ranges found 6 to 13 particles in each sample and there is a significant difference in MP particle concentration ($p<<0.05$) in these three size ranges shown in Figure 16.

**Figure 16: MP particles distribution according to the size ranges while using different of Nile Red solution in samples**

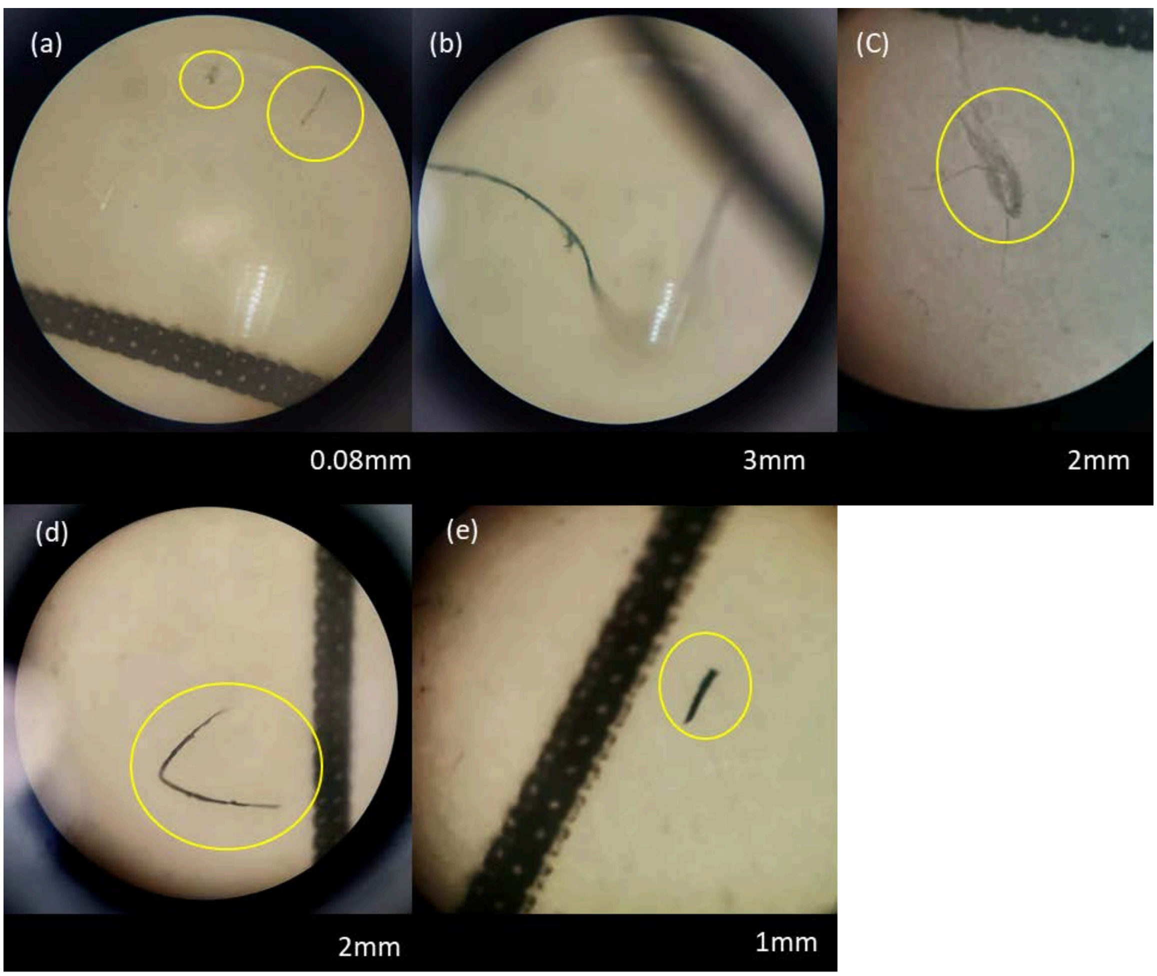



The figure 16 represented the mean and standard deviation each bar and error bar. Different color (black and gray) assigned for resenting the different concentration of NR solution used in samples. MPP abundance is found in the size ranges of 0.3mm-0.5mm for 20% NR and size range of 0.5mm-1mm for 10% NR solution in samples.

## 8.6 Shapes of Microplastics

Based on the shapes, four groups were detected such as microbeads, fiber, fragments and films. In the optical microscopy analysis, most of the particles were identified as fibers followed by films and pallets shown in Figure 17. Moreover, in the fluorescence microscopy analysis, the dominant detected particles were microbeads followed by the fibers and fragments shown in Figure 18 and Figure 19.

**Figure 17: Different shapes of microplastics observed in water bottle samples under optical microscopy analysis. The morphology is categorized as long and short fragments (a and e) , long fiber (b), short fiber (d), and palette (c) .**



In terms of microbeads and fibers detected in Brand No 7 and Brand No 3 shown in figure 18 and 19. Among the Microplastics, around 36% of particles were microbeads and 33% fibers, followed by fragments 20% and 9% films and 1% foams and others sphere shaped microbeads are mostly observed on fluorescence microscopy analysis since the size ranges mostly measure ≤1mm.

**Figure 18: Different size of microbeads observed in water bottle samples under fluorescence microscopy analysis denoted as (a-c)**

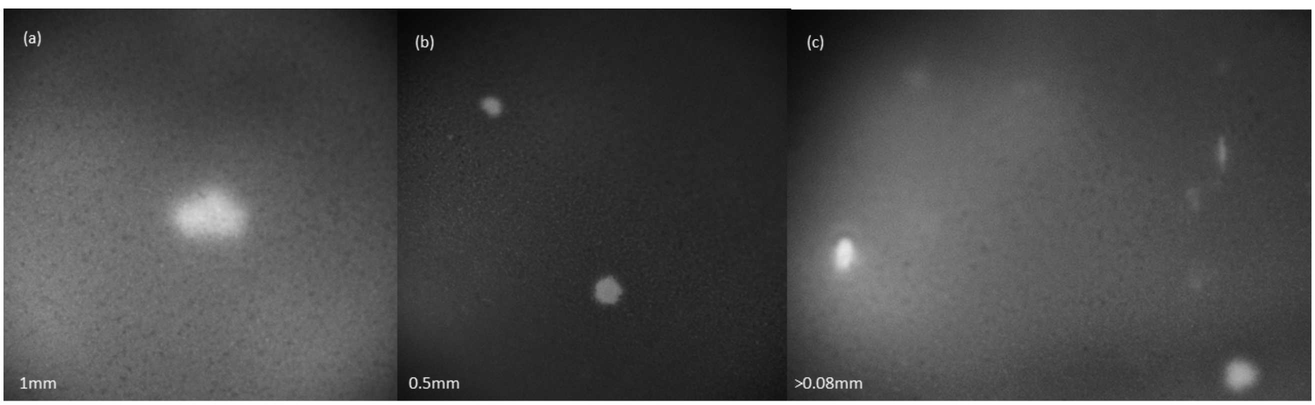


**Figure 19: different shapes of microplastics observed in water bottle samples under Fluorescence microscopy analysis. The morphology is categorized as fragments (a and b) , long fiber (c), film (d), palette (c) , and short fiber (f).**

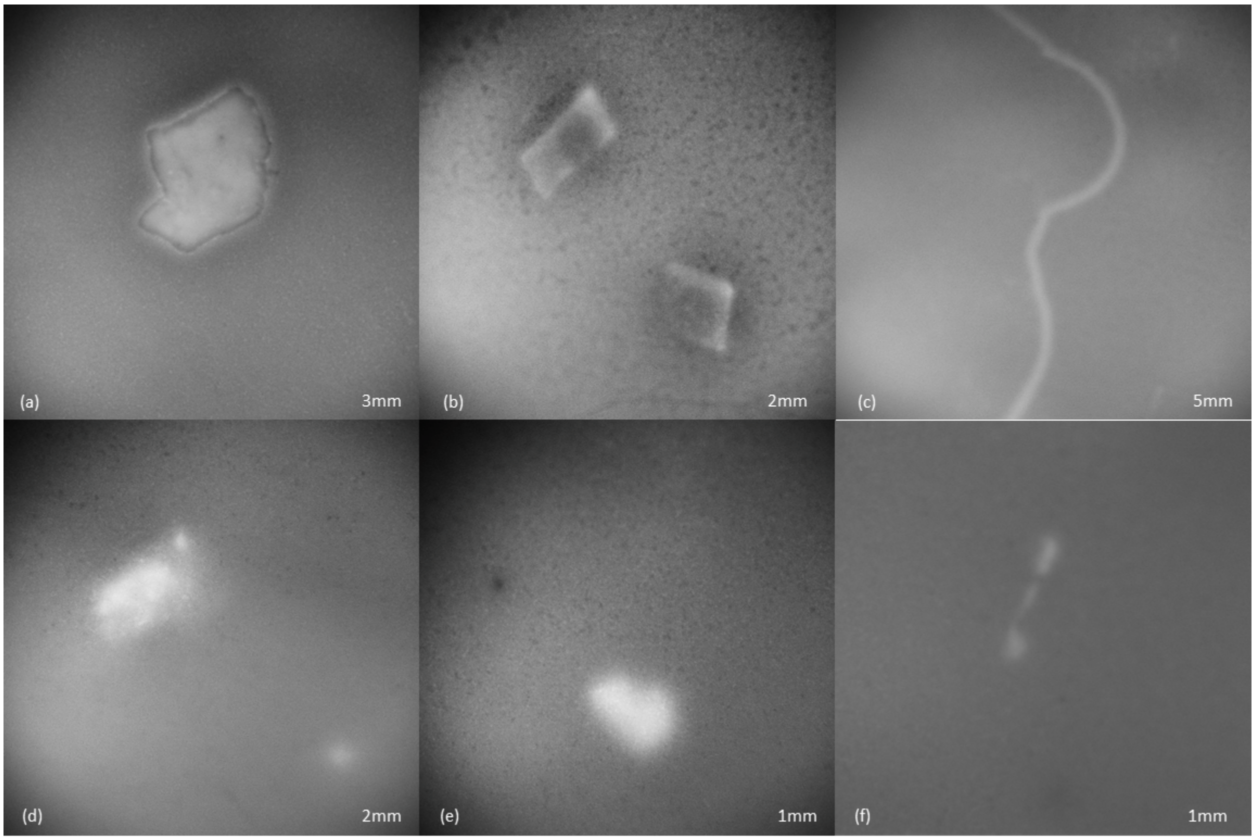
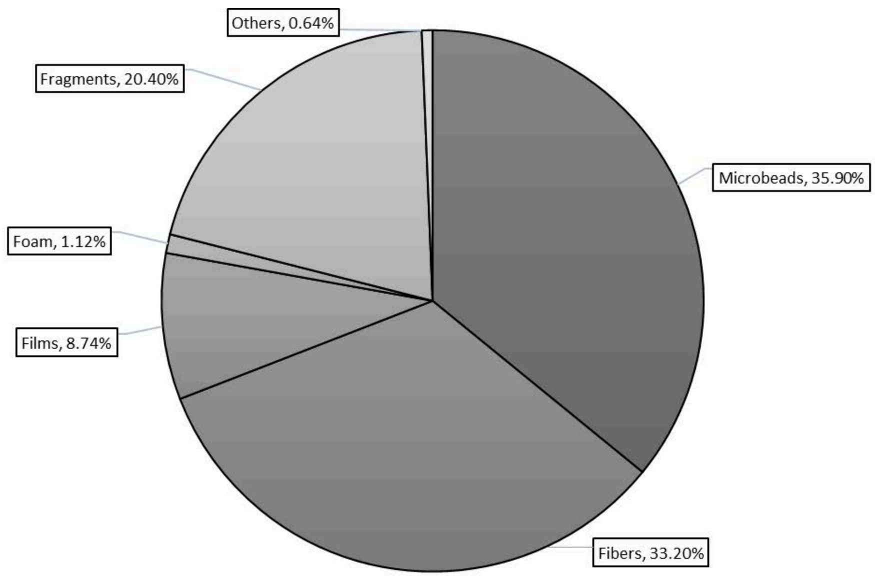


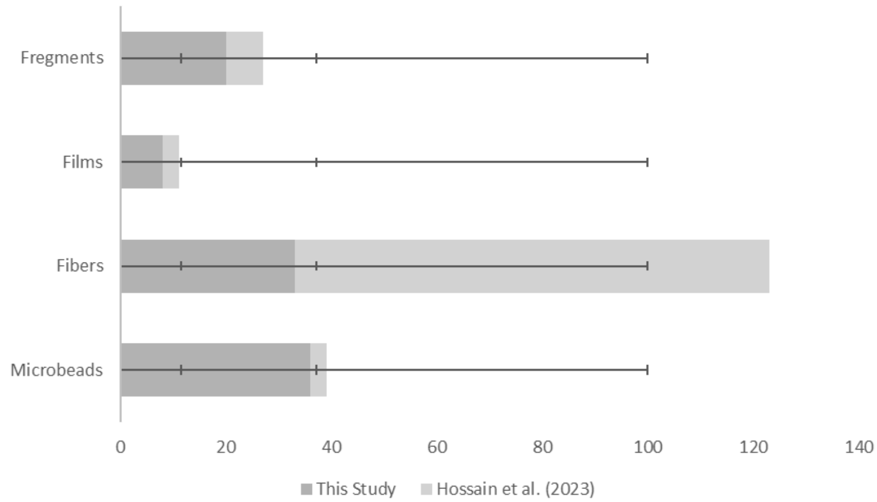
**Figure 20: Percentage of different shapes of microplastics in plastic water bottle samples**




Furthermore, a similar investigation observed by Hossain et al. (2023), shown in Figure 21 where they reported 90% fibers and 7% fragment in the bottled water samples along with 3% foams and films. Furthermore, the study of Hossain et al. (2023) reported MP in water bottles, manufactured in Dhaka city, the similarity found in their study and the dominance of fibers, beads and fragments shown in Figure 21.

However, Pivokonsky et al. (2018) reported the most abundance of plastic fibers found in tap water due to the often coming from washing machines. The study of Hossain et al. (2023) hypothesized that fibers detected from bottle water might be acquired from the unrefined water sources (Zhou et al., 2021).

**Figure 21: Morphology Based MP Concentration Fraction of bottled water in two studies in Dhaka city.**



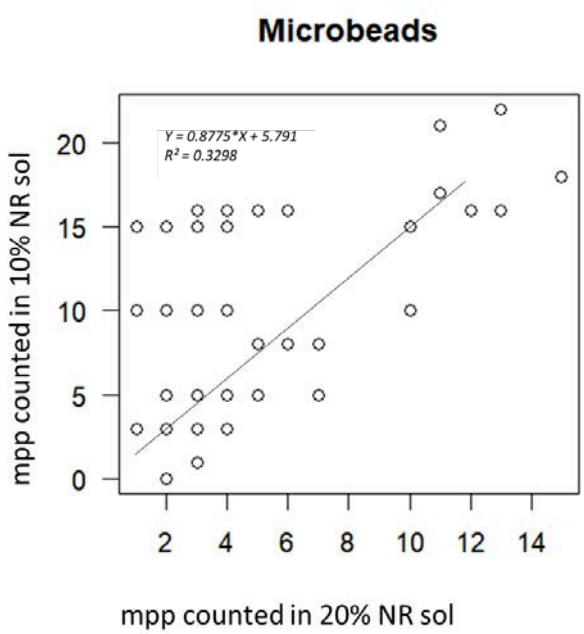

## 8.7.1. Distribution of microplastics shapes observed in different concentration of Nile Red Solution

Fluorescence stain Nile Red is a component which is 9 diethylamino-5H benzo[alpha]phenoxazine 5-one is an excellent dye for detecting microplastics particles in fluorescence microscopy through photography (Greenspan et al.,1985). In this study, image analysis through ImageJ software which allows fluorescent particles to identify and count and measure its length. Therefore, shapes and length of each particle were measured by magnified images and recorded the whole filter area. The plastics substances were viewed (excitation, 450-500 nm; emission greater than 590nm) based on surface polarity characteristics of detection particles (Maes et al., 2017). However, the number of particles is decreasing according to their sizes. Simultaneously the difference between the particles detected while using Nile red solution 20 µg/ml stock solution per sample and 10 µg/ml stock solution per sample increased. The correlation between the number of mpp observed in NR 20µg/ml stock solution and NR 10 µg/ml stock solution were identified for each sample and shape respectively.

### 8.7.2. Distribution of Microbeads in different concentrations of NR stock solutions

The microbeads, this is more pronounced than for fibers. However, the correlation coefficients were >0.5 showing moderated correlation for all tested relationships shown in Graph 04.

**Graph 04: Scatterplot with regression model considering microbeads plastics particles counts in different concentrations of NR stock solution per sample.**

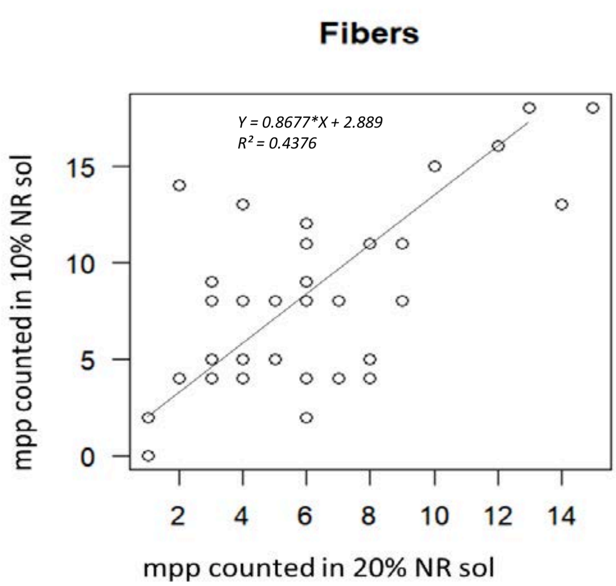


scatterplots with regression lines were produced for the different NR stock solution MPP counts for microbeads, fibers, fragments. These plots display strong explained variability according to Cohen, 1988. For microbeads, the slope of the regression line is nearly 0.9, differing significantly from zero.

### 8.7.3.Distribution of Microbeads in different concentrations of NR stock solutions

The same phenomenon was observed for fibers except for fragments shown in graph 05 and 06. However, the x-axis determined the microplastics particles detected in NR stock and the y-axis determined the microplastics detected in NR in these figures. The proportion of mp increased with decreasing NR particles and is close to zero. The statistical value showed a moderated linear regression line for fibers. The slope for the regression line is 0.8677.

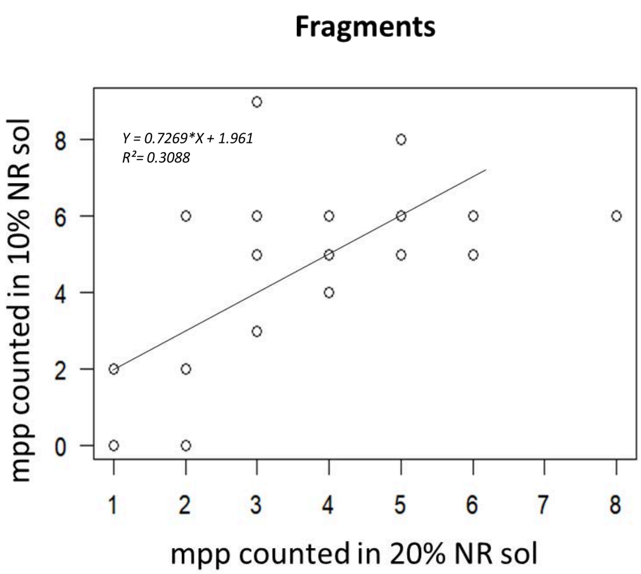
**Graph 05: Scatterplot with regression model considering fibers and plastics particles counts in different concentrations of NR stock solution per sample.**



### 8.7.4 Distribution of fragments  in different concentrations of NR stock solutions

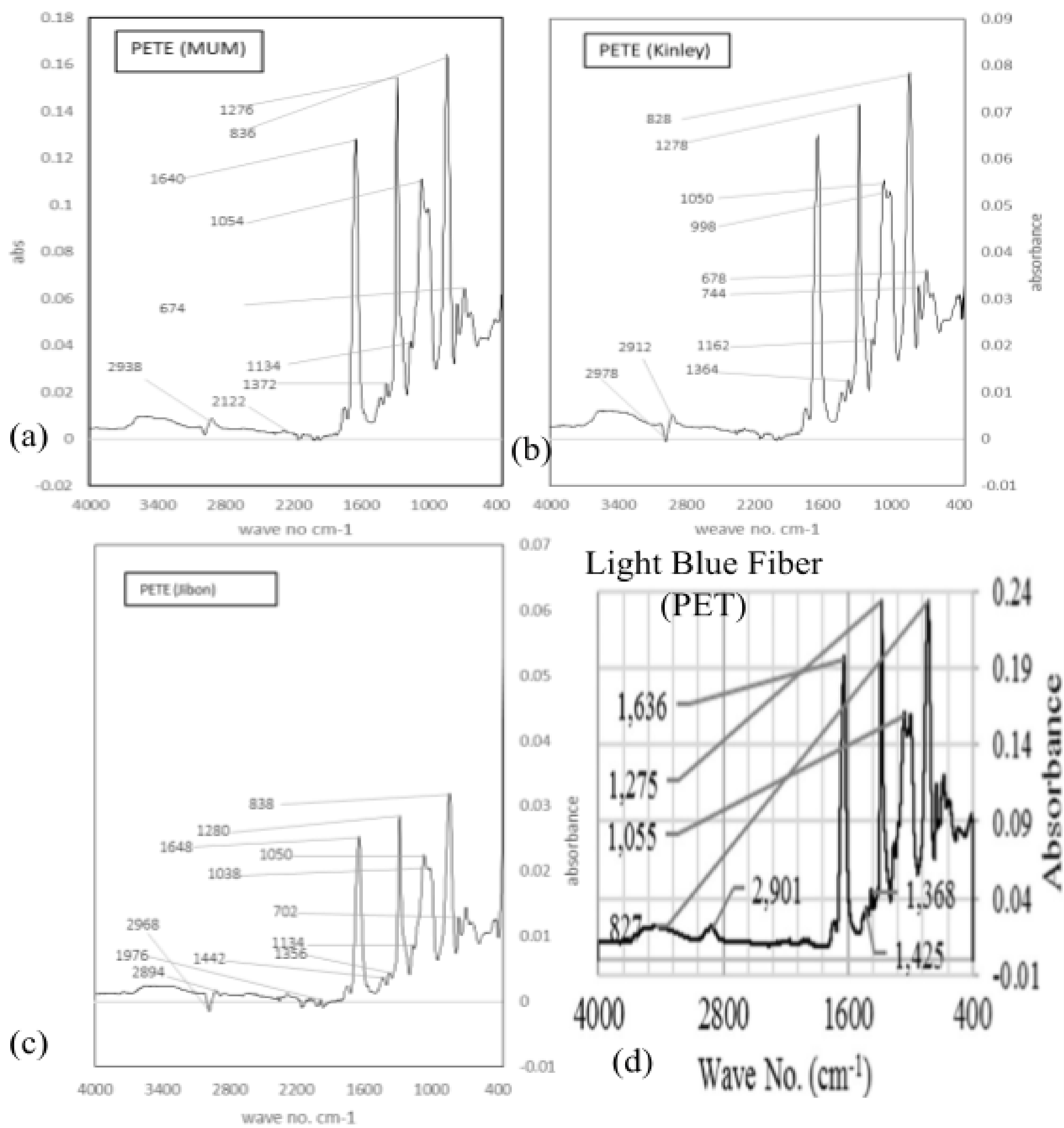
The statistical value showed a weak linear regression line for fragments.

**Graph 06: Scatterplot with regression model considering fragments of plastics particles counts in different concentrations of NR stock solution per sample.**



## 8.8 Polymer Characterization

**Graph 07: Fourier Transform Infrared (FTIR) Spectra of MPs in bottled drinking water.** (a-c) denoted from different Brands and (d) denoted reference graph from reference; Kankanige and Babel, 2020)



In the figure shown the analysis of FT-IR that identified Polyethylene terephthalate (PET) as the dominant type of polymer due to all the bottles being manufactured with the same polymer. Although the original spectral (0 to 6000 $cm^{-1}$) and sub spectral (400-4000 $cm^{-1}$) are very critical. The IR- spectrum of PET sample revealed in graph 06, the moderated peaks at 828$cm^{-1}$, 1055$cm^{-1}$, 1275$cm^{-1}$, 1368 $cm^{-1}$, 1425 $cm^{-1}$, 2901 $cm^{-1}$. Respectively, showing C-H stretch and bend was observed by absorbance at wavelengths. These values were closer to our reference value (figure d.) that we hypothesized that the bottle's main polymer was PET, which was the reason to identify the pet spectra (98%) in all the samples.

## 8.9 Survey Data Analysis

all the data from the survey was gathered that was summarized and examined. The frequency of individual data from participants was given the distinct results and comparison between them according to their age, water consumption, plastic bottles use and reused factors was given the depth of the study.

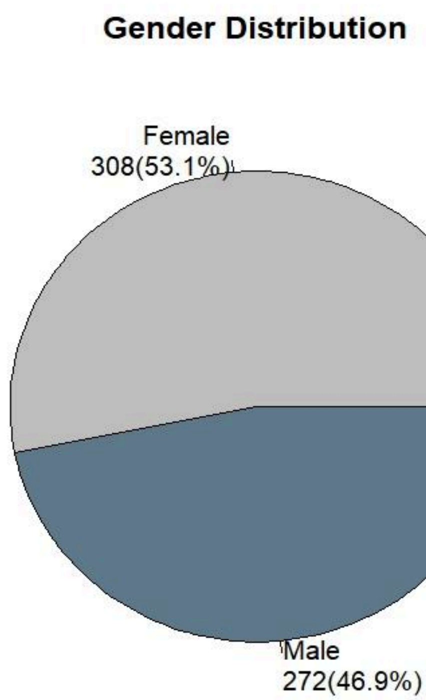
### 8.9.1 Distribution of Participants Gender

**Table 05. Frequency Table Distribution of Participant's Gender**

| | Category | Frequency | Percentage.Freq | Valid_Percentage.Freq | Cumulative_Percentage |
|---|---|---|---|---|---|
| **Female** | Female | 308 | 53.1 | 53.1 | 53.1 |
| **Male** | Male | 272 | 46.9 | 46.9 | 100.0 |

According to data presented in table 05, a cross-table was analyzed which conducted the distribution of participants' gender who used plastic bottles, without plastic bottles and reused bottles while drinking water in day to day life. The total participants was 580 where 53.1% were women ( 308 women) and 46.9% were male (272 men).

**Figure 22: Distribution of Participant's Gender**



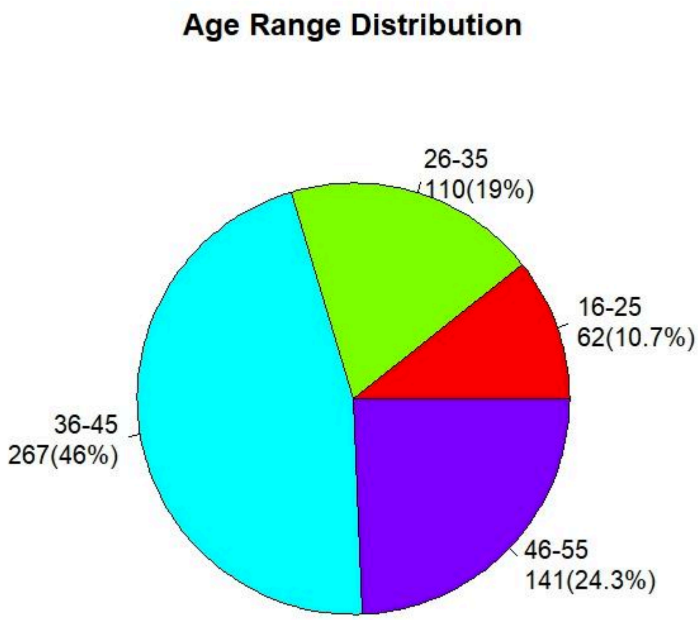

## 8.9.2 Distribution of Participants Based on Age

**Table 06. The Frequency Table Distribution of Participants Based on Age**

| | Category | Frequency | Percentage.Freq | Valid_Percentage.Freq | Cumulative_Percentage |
|---|---|---|---|---|---|
| **16-25** | 16-25 | 62 | 10.69 | 10.69 | 10.69 |
| **26-35** | 26-35 | 110 | 18.97 | 18.97 | 29.66 |
| **36-45** | 36-45 | 267 | 46.03 | 46.03 | 75.69 |
| **46-55** | 46-55 | 141 | 24.31 | 24.31 | 100.00 |

In table 06, the table contained the age variation between the participants who were drinking water bottles . The study showed that 46.03% of people whose ages are between 36-45 participated in the online survey.

**Figure 23: Distribution of Participants Based on Age**




### 8.9.3 Distribution of Participants' Occupation

**Table 07. The Frequency Table Distribution of Participants' Occupation, based on reused plastic bottles**

| | Category | Frequency | Percentage.Freq | Valid_Percentage.Freq | Cumulative_Percentage |
|---|---|---|---|---|---|
| **Employed** | Employed | 221 | 43.68 | 43.68 | 43.68 |
| **Freelancer** | Freelancer | 116 | 22.92 | 22.92 | 66.60 |
| **Student** | Student | 59 | 11.66 | 11.66 | 78.26 |
| **Unemployed** | Unemployed | 110 | 21.74 | 21.74 | 100.00 |

In this table 07, it contained distribution according to the participants' occupation who used plastic bottles repeatedly (more than one time) Among 580 participants, 506 plastic bottles reuse the plastic bottles while consuming and storing drinking water. It was found that most of the participants who were employed tended to store water in plastic bottles which was 43.68% while the decreasing number of students who used plastic bottles was 11.66%. Freelancer and unemployed participants were reusing plastic bottles nearly 23% and 22% respectively. In the frequency table we excluded the participants who did not reuse plastic bottles.

**Figure 24: Distribution of Participants' Occupation, based on reused plastic bottles**

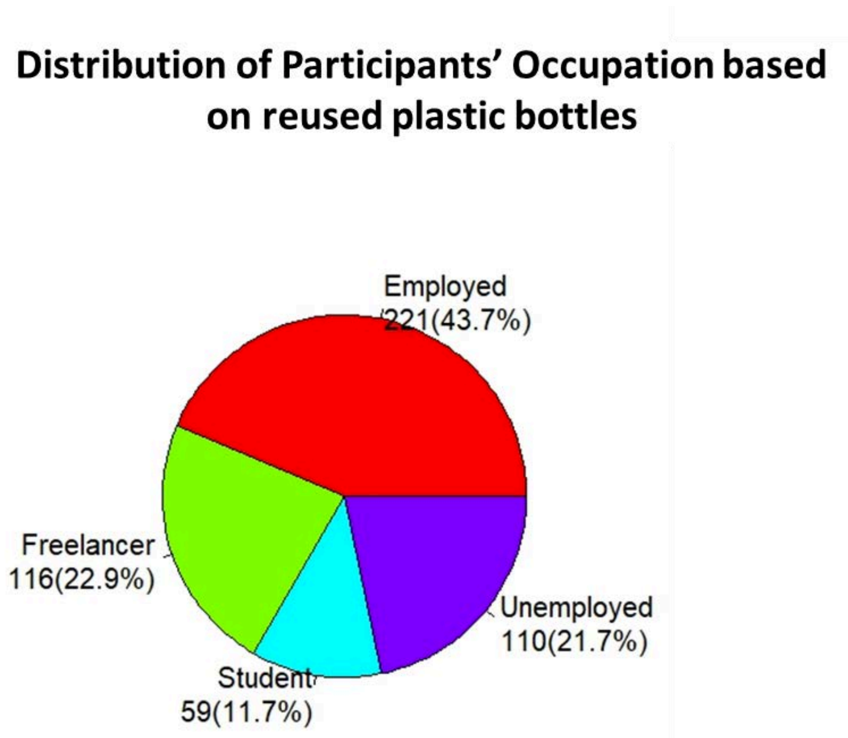
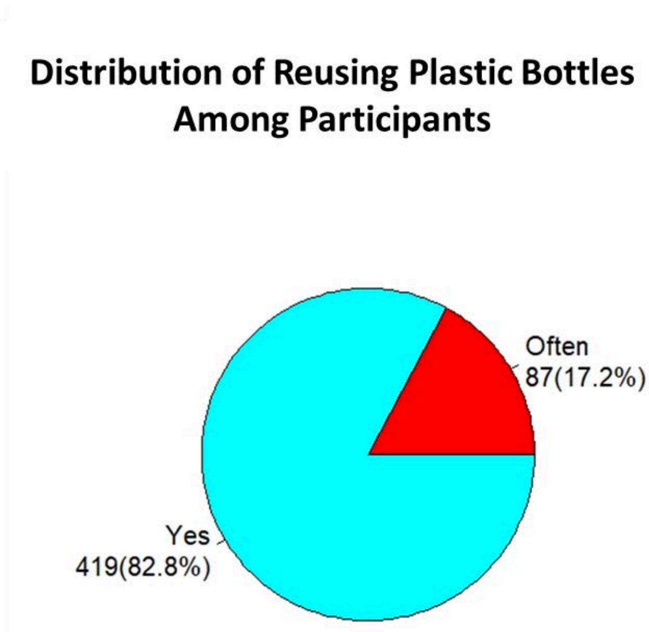


**Figure 25: Distribution of Participants' based on reused plastic bottles (excluding answer "No")**



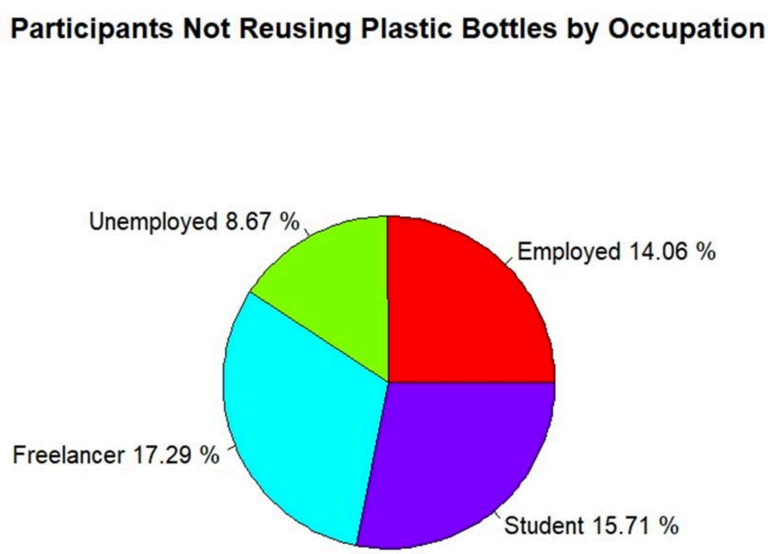

### 8.9.4 Distribution of Participants' Not Reusing Plastics Bottles By Occupation

**Table 08. Distribution of Participants' Not Reusing Plastics Bottles By Occupation**

| | Occupation | Count | Frequency | Percentage | Cumulative_Percentage |
|---|---|---|---|---|---|
| 1 | Employed | 21 | 0.2837838 | 28.37838 | 28.37838 |
| 2 | Unemployed | 23 | 0.3108108 | 31.08108 | 59.45946 |
| 3 | Freelancer | 19 | 0.2567568 | 25.67568 | 85.13514 |
| 4 | Student | 11 | 0.1486486 | 14.86486 | 100.00000 |

Table 08, contained the participant's who usually do not use plastic bottles to store drinking water. Among 74 participants around 14.06% were employed, followed by 8.67% being unemployed, 17.29% freelancer and only 15.71% were students. The data interpreted that maximum participants who were students did not reused plastic bottles whereas, the most employed participants used plastic bottles.

**Figure 26: Distribution of Participants' Not Reusing Plastics Bottles By Occupation**



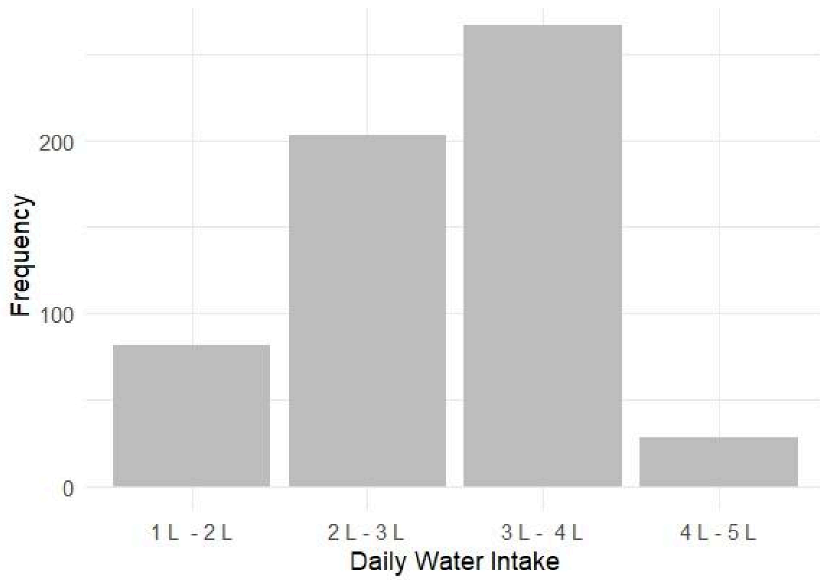

### 8.9.5 Distribution of Participants' Daily Intake Water

**Table 09. Table Distribution of Participants' Daily Intake Water**

| | Daily_Water_Intake | Frequency | Percentage | Cumulative_Percentage |
|---|---|---|---|---|
| 1 | 1 L - 2 L | 82 | 14.14 | 14.14 |
| 2 | 2 L - 3 L | 203 | 35.00 | 49.14 |
| 3 | 3 L - 4 L | 267 | 46.03 | 95.17 |
| 4 | 4 L - 5 L | 28 | 4.83 | 100.00 |

The table 09, contained information that around 46% participants consumed 3 to 4 liters. The average daily water intake according to our survey was 3.5 liters. While the 35% participants drinking water was 2 to 3 liters.

**Graph 08. Distribution of Participants' Daily Intake Water**




## 8.9.6 Distribution of Water Bottle Purchase by Participants

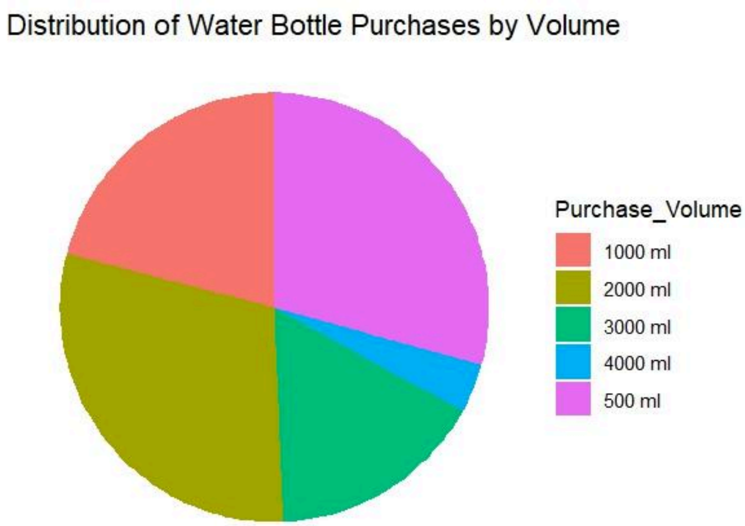
**Table 10. Cross-Table Analysis Distribution of Water Bottle Purchase by Participants**

| | 1000 ml | 2000 ml | 3000 ml | 4000ml | 500ml | Total |
|---|---|---|---|---|---|---|
| Employed | 27.9% | 28.5% | 9.2% | 4.1% | 30.3% | 100% |
| Freelancer | 16.1% | 14.5% | 13.7% | 2.4% | 53.3% | 100% |
| Student | 31.9% | 46.4% | 11.6% | 0.0% | 10.1% | 100% |
| Unemployed | 7.8% | 37.5% | 33.5% | 6.3% | 14.9% | 100% |

Table 10, provided the information that the participants who were employed purchased 500ml to 2 liters of water most, while the freelancer participants mostly purchased 500ml bottles of water.

Students often bought 1 to 2 liters while unemployed participants purchased 2 to 3 liters of bottled water according to the survey.

**Figure 28: Distribution of  Water Bottle Purchase by Participants**



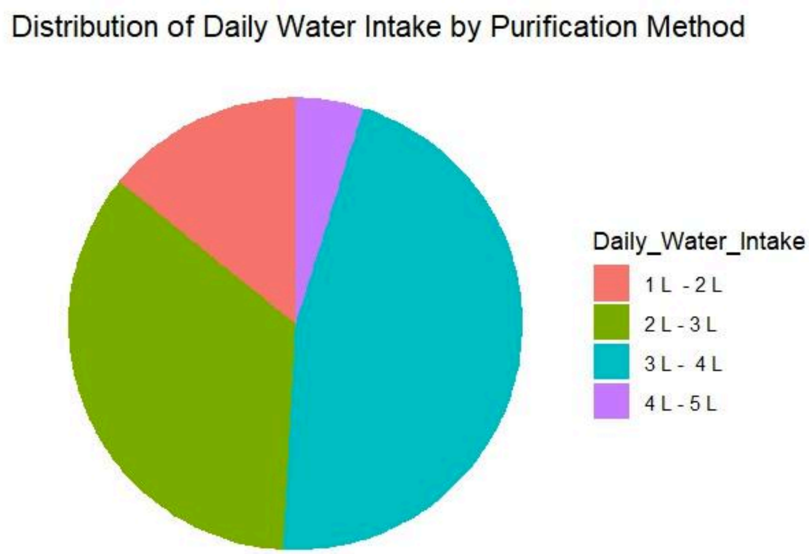

### 8.9.7 Distribution of  Water Volume purification method used by Participants

**Table 11. Cross-Table Analysis Distribution of  Water Volume purification method used by Participants**

| | 1 – 2 L | 2 – 3 L | 3 – 4 L | 4 – 5 L | Total |
|---|---|---|---|---|---|
| Boiled Water | 12.9% | 37.6% | 46.0% | 3.5% | 100% |
| Filtered Water | 12.9% | 35.2% | 47.8% | 4.1% | 100% |
| Purchase Bottle Water | 14.2% | 31.3% | 48.5% | 6.0% | 100% |
| Raw Water | 17.7% | 52.9% | 29.4% | 0.0% | 100% |
| Tap Water | 20.0% | 30.8% | 41.5% | 7.7% | 100% |

This table 11 showed that most of the participants purified water through the boiled method and filtered method and the volume of water was 3 to 4 liters.

**Figure 29: Distribution of Water Volume purification method used by Participants**




## 8.10 Estimation of dietary intake of microplastics

Microplastics are undoubtedly harmful for human health. In the chapter we measured dietary intake measurements to evaluate an individuals and populations drinking water consumption through our survey report. The estimation of daily intake (DI) of Micro plastics from drinking water bottle was evaluated through this equation:

DI = (C x WC)/ average body weight (Tse et al., 2022)

C represents the concentration of MPPs (particles per L) detected in our study, WC represents water consumption per day. Our study identified an average rate of microplastics abundance is 33.33p/L, while, average of >1 mm sized mpp which is 10.683 p/L particles per liter per filter and average of <1 mm size mpp which was 22.87 p/L. According to statistical data from our survey, the average volume of bottled water consumption in Dhaka City was 3.5 liters per day measured as WC in per day with the assumption only bottled water is the main source of water. According to our survey the average body weight (kg) of Dhaka city with age ranging from 16-55 above was 62 kg. As shown in table 12, calculate the dietary intake of mpp per day and also in year.

**Table 12. Estimation of dietary intake of microplastics**

| | *Bottled Water* | *Calculated DI of MP* |
|---|---|---|
| | *3.5 L* | |
| *Body Weight (kg)* | *62kg* | *1.89p/L* |
| *Average of >1 mm size particles per day* | *10.683 p/L* | *0.60p/L* |
| *Average of <1 mm size particles per day* | *22.87 p/L* | *1.29p/L* |
| *Total particles per day* | *1.89 p/L ~ 2p/L* | |
| *Annually Total* | *689.85p/L~ 730p/L* | |

Table 12 analyzed the DI/ Dietary intake of microplastics based on a survey, using the mean value of different size ranges of MP particles, we predicted that an adult intake of mp particles is nearly 2 particles per Liter of water consumption per day. And annually it estimated 730p/L particles per liter per year.

## 8.10 Overview of individual papers reporting on microplastic

**Table 13. Overview of Individual Papers Reporting Microplastic Characteristics and Detection Methods in Bottled Water**

| Year | Reference | Packaging | Analytical Method | Shape | Detected Polymers | Abundance |
|---|---|---|---|---|---|---|
| 2024 | This Study | PET bottles, Glass | Fluorescence Microscopy | Beads, Fibers, Fragments, Films | PET | 33.33 ± 10.4 |
| 2024 | Zhu et al. (2024) | PET bottles | - | Fragments, Fibers | PET, PP, PE | 38.45 p/L |
| 2023 | Hossain et al. (2023) | Recyclable plastic bottles | FTIR | Fibers, Fragments, Films, microbeads | LDPE, HDPE, EVA, PETE | 35 ± 19 item/L |
| 2022 | Li et al. | Plastic bottles | FTIR | Fragments, Films, Fibers, Foams | PP, Nylon, PS, PE, PEST | 10 item/L |
| 2021 | Weber et al. | - | - | - | Cellulose, PETE | 113 ± 83 item/L |
| 2021 | Almaiman et al. | PET, Glass, PCTE | FTIR | | PE, PS, PET, PP, PA, PU | From 0.99 -4.2 p/L |
| 2021 | Weisser et al. | Glass | FTIR | Fragments | PE, PS | 317 ± 257 p/L |
| 2021 | Zhou et al. | PETE | RM | Fibers, Fragments | PP, PS, PE, PETE, PU, PVC, PA, PAA, PEVA, cellulose | 2–23 particles/bottle |
| 2020 | Kankanige et al. | PET, Glass | FTIR RM | Fibers, Fragments | PET, PE, PP, PA | 140 ± 19 p/L |
| 2019 | Winkler et al. | PET | SEM-EDS | - | PET. PE | 148 ± 253 p/L |
| 2019 | Zuccarello et al. | PET | SEM-EDS | - | - | $5.42 \times 10^7$ p/L |
| 2018 | Mason et al | PET, Glass | FTIR | Fragments, Film, Fiber, Foam, Pellet | PP. NY. PS. PE PEST | 315 p/L |
| 2018 | Obmann et al. | PET, R-PET, Glass | RM | Fragments Fibers Films | PET, PE, PP | 2649 p/L |
| 2018 | Schymanski et al. | PET, R-PET, Glass, Carton | RM | Fragments, Fibers | PEST, PE, PP, PA, others | 14 ± 14 p/L (PET) |
| 2016 | Wiesheu et al. | PET | RM | Fibers | PET | 1 particle |

PET was the most studied packaging in fifteen studies in 2016 to 2024 shown in table 13 and 14, three studies investigated microplastics in repeat packaging whereas, glass packaging also investigated in seven studies. Lastly, cartoon and PCTE packages were found in only one study.

MP size and particles investigated specifically by Oßmann et al. that reported smaller than 0.1mm mpp.

**Table 14. Overview of Individual Papers Reporting Microplastics Size Range in Bottled Water**

| Year | Reference | Size |
|---|---|---|
| 2024 | This Study | >0.08– 3mm |
| 2024 | Zhu et al. (2024) | <5 µm |
| 2023 | Hossain et al. (2023) | 5 – 100 µm |
| 2022 | Li et al. | 5- 100 µm |
| 2021 | Weber et al. | <5 µm |
| 2021 | Almaiman et al. | 25-500 µm |
| 2021 | Weisser et al. | 11 -500 µm |
| 2021 | Zhou et al. | - |
| 2020 | Kankanige et al. | ≥ 6.5 µm |
| 2019 | Winkler et al. | ≥ 3 µm |
| 2019 | Zuccarello et al. | 0.5 -10 µm |
| 2018 | Mason et al | 6.5 -100 µm |
| 2018 | Obmann et al. | 1-10 µm |
| 2018 | Schymanski et al. | 5 – 1359 µm |
| 2016 | Wiesheu et al. | - |

According to the table 13 and 14 , 10 micrometers, smaller microplastics participles were only looked at by Oßmann et al. and Zuccarello et al., who found an average concentration of 2,649 particle per litter for PET packaging and $5.42 \times 10^{7}$ p/L for other packaging. Mason et al. only evaluated MPs greater than 100 micrometers with an average concentration of approximately ten particles per litter. In total, 5 of the investigations assessed MPs across a wider or differing measurement size range. A concentration of 315 particle per litter was observed within a measurement size range of 6.5 to 100 micrometers; within a size range of 5–1359 micrometers, a concentration of 14 ± 14 p/L was noted; for particles between ≥ 3 micrometers, the concentration was 148 ± 253 p/L; for MPs ≥ 6.5 micrometers, the concentration was 140 ± 19 p/L; and for a size range of 25–500 micrometers, concentrations ranged 0.9 - 4.2 p/L. Only a single MP was identified by Wiesheu et al., and no details are available as to size.

MPs were narrated for both glass bottles and expectations. According to Oßmann et al.,per liter there were an average of 5864.1 particles smaller than 10 micrometers per liter. The particles found by Mason et al. were bigger than 100 micrometers, and there were 8.96 particles per liter of fluid.

There were fifteen studies conducted on MPs, including this one, within a broader range of MPs size shown in figure 14 and 15. Mason et al. recorded a concentration of 195 p/L for particles from 6.4-100.um. Obmann et al. obtained a preoccupation of 434.1 p/L for particles over 10 micrometers; Schymanski et al. established that the concentration was 50± 52 p/L with the smallest and largest MPs sizes being 5 – 1359 micrometers. Weisser et al. obtained a preoccupation of 52 ± 4 p/L for particles from 6.5.um in size. Kankanige et al. took samples of MPs from 11-500 micrometers in size and found the concentration to be 317 ± 257 p/L. Conversely, Almaiman et al. did not find any particles in their analyzed samples. The presence of MPs in R-PET packaging was only considered in the studies by Obmann et al. and Schymanski et al.. Also, only the size distribution of MPs below 10 micrometers was determined in this study, however, the MPs sizes were only analyzed in the study by Oßmann et al. who noted the MP below 0.1 mm MPP.

# Chapter 09

## Discussion

## 9.1 Introduction

The chapter contained the discussion on finding from fluorescent microscopy analysis on microplastics contamination in bottled drinking water in Dhaka city with the limitation of the study and the potential research idea plans developed from the study and finding to enhance the work in future. Therefore, the overall objective of study was to identify and characterize microplastics in samples collected from common, popular brands at local stores. The observation of the research and quantifying the microplastics debris or items used Fluorescence Microscopy analysis and Image J software. To characterize the morphology of polymers used the FT-IR analysis.

Different size ranges are measured with morphology of mp taken in this study and the samples are individually analyzed (n=36) , including pet bottles and glass bottles where mp particles were detected in  all the samples . microbeads, fibers, fragments are prominently observed in this study, however, film, pellets and other shapes almost were not noticeable. The size of MPP has higher concentration in <1mm due to the shear packaged qualities of bottles hypothesized(Hossain at el, 2023).. Based on the characterization PET found in all studies and countries including USA, Germany, Hong Kong, Thailand, Korea, Bangladesh and so on.

Few studies had been documented on MP pollution or contamination in human consumables in 2016 to 2024 specifically drinking water such as  Oßmann et al , Mason et al, Schymakanski et al, Hossain et al,   and so on. Different studies analyze MPP in several methods that are expensive and time consuming. In contrast, to analyze drinking water samples, our study act towards acknowledgement used the method which is cost effective, reliable  which could be quickly done  and efficient. Here, Nile Red using in all samples were analyzed for the presence of microplastics in different size range, as NR solution can detected the particles <0.025mm (Mason et al.,2018)

## 9.2 Abundance of microplastics

In this study, the MPP identified (average MPP per filter) 33.33 ± 10.4 MPP/L and there is a significant difference ($p<<0.05$) while using two different concentrations of NR stock solution in samples. In Bangladesh the similar observation was found in Hossain et al. 2023 which was 23 ± 19 MPP/L. In addition, Schymanski et al. found 50± 52 MPP/L in terms of the smallest size of microplastics . However, Mason et al. observed nearly two hundred particles starting from the size 0.064 to 0.1mm. The increasing amount of microplastics detected to the proportion of decreasing the size range was observed through these researchers including our study. Due to the Microplastics contamination , humans are gradually exposed to MP particles by ingesting food, beverage and water and their compounds have toxic elements that have been associated with the humans, animals and ecological assessments (Impacts of Microplastics on Health (Signal), 2024).

## 9.3 Sizes of Microplastics

Recovered microplastics detected from plastic bottle water were classified into five size categories : 3mm-5mm, 1mm-3mm, 0.5mm-1mm, 0.3mm-0.5mm, 0.08mm-0.3mm. None of the microplastics were detected that were larger than 2mm in size. Above all, 45% and 63% of the particles were greater than 1mm while using 10 µg/ml NR stock solution per sample and 20 µg/ml NR stock solution respectively. On the other hand, 55% and around 70% of the particles were less than 1mm in size. Therefore, two different concentration NR stock solutions were used to accurately assess the results of this research. However there was a limitation to identify the nanoplastics due to the method we applied. To cope with the limitation several studies used RM spectroscopy and Fluorescence microscopy with x10, x20, x50 magnification for measuring smallest plastics pieces (Schymanski et al). Cataroteis are: 5-10 mm, 10-20mm, 50-100mm and greater than 100mm. For example, Oßmann et al observed ninety percent of microplastics were sized in below 0.05mm found in mineral water. In addition, sized in between 0.065mm is 95% of microplastics observed in Mason et al. and Hossain et al.

## 9.4 Morphology of Microplastics

In this research four types of microplastics identified in terms of their shape such as: beads, fibers, fragments, films. Most of the observed microplastics were microbeads. In the optical microscopy analysis, most of the particles were identified as fibers followed by films. Moreover, in the fluorescence microscopy analysis, the dominancy detected particles were microbeads followed by the fibers and fragments. In terms of microbeads and fibers detected in Brand No 7 and Brand No 3 which was 64 ± 17 MPP/L. Among the Microplastics, around 36% of particles were microbeads and 33% fibers, followed by fragments 20% and 9% films and 1% foams and others . A similar investigation observed by Hossain et al. (2023) where they reported 90% fibers and 7% fragment in the bottled water samples along with 3% foams and films. Furthermore, the study of Hossain et al. (2023) reported MP in water bottles, manufactured in Dhaka city, the similarity found in their study and the dominance of fibers, beads and fragments.

## 9.5 Characterization of Microplastics

Regarding plastic polymers PETE was the most common polymer along with PE in bottled water. PET could be originated from the packaged itself and bottleneck and PE could be derived from the bottle cap. However, in tap water PE and PET were most common polymers detected in several reports. In this regard, the analysis of chemical components was important to discriminate factors in terms of sizes and MP abundance. We analyzed the samples in the FT-IR spectroscopy method and observed the transmittance. For better understanding, transmittance is converted into absorbance and plots the graphs in ggplot (R studio programming) where the IR- spectrum of PET sample revealed in the peaks at 827cm$^{-1}$, 1055cm$^{-1}$, 1275cm$^{-1}$, 1368 cm$^{-1}$, 1425 cm$^{-1}$, 2901 cm$^{-1}$.

## 9.6 Human Health and Dietary Exposure of Microplastics

According to the study, intake of mp particles is nearly 2 particles per Liter of water consumption for an individual. And annually it is estimated around 730 particles per liter which is harmful for human health. Several studies show the statistics where it was found that the average level of microplastics on foods, ingredients, water and so on. For example, Cox et al., 2019 reported 1.48 particles/g on seafood, 0.44 particles/g on sugar and 94.37 particles/L in bottled water. On average, around 39000 to 52000 microplastic particles are consumed by people per year which indicates consumption of microplastics mostly via ingestion through foods and water. (Yee et al., 2021).

## 9.7 Limitations of The Research

In the university laboratory, the best possible environment was maintained to conduct the experiment, yet some of the obstacles were encountered. These limitations were carefully managed to get the maximum attainable outcomes.

The research labs were not completely air tight to control the air quality. The laminar hood was used with the glass kept down, yet some of the lightweighting microplastic fibers had contaminated the samples. To calculate the impurity of the air surrounding the experiment area, two environmental controls were placed there and left it uncovered for a maximum of 10 minutes and proceeded with all the steps to detect microplastics. These environmental samples were documented on each day the samples were tested. The results obtained from the environmental controls were then subtracted from the sample results.

According to *maes/ cole* the appropriate temperature for incubation was 2 to 3 hours at 60℃. In the laboratory, the shakers had 37℃ as the incubation temperature, which was almost half of the suggested temperature, so the tested samples were kept in the shaker incubator for 4 to 5 hours. The purpose of maintaining a proper time and temperature was to intensify the pigmentation of microplastic particles. As the temperature and time was modified, the results were slightly different from the references.

The suggested water for the experiment was ultrapure water. This water is free from contaminants and highly pure. Because of the unavailability of this water in the university laboratory, DNase/RNase-free water was used for the experiment.

Fourier Transform Infrared Spectroscopy (FTIR) and Raman Spectroscopy are two preferable methods to characterize the plastic particles. For this research experiment, FTIR was used to characterize the microplastics but Raman spectroscopy can detect.

## 9.8 Prospects of Future Research

In this thesis, we studied samples of drinking water from different brands including Bangladeshi and international brands. The quantity of sample allowed us to perform an in-depth study of MP in drinking water in different concentrations of NR solution, at different time duration, to draw general conclusions about the population of MP particles as fibers, pallets, beads, filaments and so on. Therefore, the different concentrations of NR determined the mp with better visibility. Bottled water contained mostly PET and PP which were then labeled with NR dye and filtered with Filson Sterile Membrane Filter papers. So, the results of the study revealed a range from 450 to 560 nm at which the membrane filter papers exhibited luminescence of the particles. Additional research revealed that Nile Red also works to dye irregular shaped microplastics and plastic particles. The Acetone rinse has not visibly damaged the microfibers. Most uptake studies to date have used fluorescently labeled microspheres obtained from specialty manufacturers simply as a tracking tool, showing that microplastics are stuck to or ingested. Given the use of NR enables fluorescent labeling of microplastics with different shapes, a wider diversity in future experimental designs is predicted to utilize alternative forms for plastic. We observed in this study that the visibility of the MP particles (mostly beads) was more prominent on the 20µl NR dye solution and incubated for 3 hr, whereas, the less concentrated solution with 10µl NR and incubated for 5 hr, thus visualized the vague outline of the particles on the fluorescence microscope, the Olympus ix73 Microscope.

Recently, the research used vibrational spectroscopy, electron microscopy and chemical or physical characterisation, the study of polymer types also reduces over-calls or under-errors as well as aids in automated looking (Maes et al., 2017). Determining the polymer composition of particles as small as a few micrometers is possible via Fourier transform infrared (FTIR) and Raman microspectroscopy. While some publications have appeared in the last years regarding automating IR-microscopy procedures for decreasing human effort and related time-budgets used to identify microplastics, these techniques are not widely applied in monitoring as their slow speed of analysis, combined with a high price that make impossible using them for processing larger ensembles of samples by it (by either type), or finally low spectral resolution. (Maes et al., 2017; Nirmala et al., 2023).

Therefore, for further on going research, to identify individual particles by their spectral fingerprints may also be difficult for microscope, especially for fine dust that are less than 20 μm in diameter as the volumetric specimen is still a necessary input of samples through IR microscopy, so that the residual measurement errors from neighboring regions or other non-targeted entities can only be solved by higher resolution. Any original polymer composition for the polymers extracted from different water bottles could come in contact with UV induced photodegradation, thermal degradation and biodegradation. Consequently, it is likely that microplastics will rapidly be covered with bacterial assemblages, which are every time plastisphere-specific. This is exacerbated by changes in the spectrum and lack of library spectra specific to polymer degradation products, as well as formation of biofilms on certain polymers or presence, absence or recognition issues with polymer additives. These are strongly accentuated in the case of fine particles, due to the high surface to volume ratio amplifying both information from this surface material rapidly obscuring the bulk signal. Hence, a very significant amount of particles will be part of an unknown category, making it difficult to isolate natural polymers like lignocellulose and chitin. Hence, despite these draw-backs, the methods that will be suitable for analysis of innumerable polymers are still FT-IR and IR spectroscopy; they will remain to be applied most significantly in investigations on small plastics particles (Maes et al. (2017; Nirmala et al., 2023).

# Chapter 10

# Conclusion

Plastics are very popular in terms of items, toys, polythene bags, and furniture in Bangladesh as there is a large portion of the national and trading market of plastics goods. Thus, there is a distinct probability of the invasion of microplastics in different kinds of packaged water, beverages through feedstock or packaging (Hossain et al. 2023). Moreover, the accessibility, morphology and exposure of dietary intake of microplastics in packaged water bottles were investigated from the south zone of Dhaka city in Bangladesh. The detection of microplastics has become crucial due to their widespread presence in the environment which is causing potential risks to ecosystems and public health. Therefore, drinking water is the most important requirement of the body which is nowadays becoming one of the primary sources of microplastics liability of humans. In contrast, several studies investigating microplastics exposure through water consumption to all over human health are still ambiguous. Another severe threat of microplastics pollution is environmental effect through smallest plastics debris from primary and secondary sources, alarming concern worldwide. Due to the tiny size of microplastics several studies reported mp from various species, even in human brains, blood, digestive system and so on. (Lamichhane et al. 2022) . Our purpose of the study is to examine the microplastics presence in water bottles from different popular brands.

This study concluded that the investigation of microplastics presence in bottled water based on the finite number of samples (n=36) through fluorescence microscopic analysis with fluorescence tagging Nile Red dye while characterizing the microplastics type through FT-IR. The observation of microplastic analysis done by optical microscopy and fluorescence microscopy while measuring the sizes conducted through image J software. As there is an emerging concern of microplastics ingestion, the research also estimated the daily dietary intake of microplastics by drinking water through an online survey where nearly 600 people submitted proven answers based on questionnaires.

The survey has done just a small reflection of the consumption rate of microplastics through water in daily life at Dhaka city of Bangladesh. In spite of the number of people of this survey contained were not sufficient to get the exact number of microplastics consumption rate individually, nevertheless, it provides the comprehend fully the average age, weight of people drinking water intake using plastic bottles, reused plastics bottles of each people. Since the survey was conducted online anonymously, all the participants were reluctant to provide their information individually. The archived responses were enough to draw a conclusion that each consumed 2 microplastics particles per day at least with the average body weight of 62 kg. The people who participated mostly employed females, who reused the purchasable plastic bottles for storing drinking water.

Through this examination, all the water brands contained more or less microplastics with an average ($23.17 \pm 7.04$) items/filter and ($33.33 \pm 10.4$) items/filter while using 10 µg/ml and 20 µg/ml NR solution in acetone respectively. Five morphology of microplastics analyzed with microbeads, fibers and fragments dominancy where the 36%, 33% and 20% respectively. Transparent microplastics and blue fiber microplastics observed through optical microscopy analysis. Among the characterizing polymers PET was dominant. The estimated daily dietary intake of microplastics particles was approximately 2 particles per liter of water per day for each person from drinking water with the probability of health hazards. Microplastics in drinking water are a matter of concern with the safety and quality of the water supply and may have major effects on public health. Further study of microplastics analysis in foods, beverages can help to understand the impact of microplastics on human body and health. To sum up, the production and packaging of bottled water required keeping under surveillance through administrator, legislator and government so that the health risk factors could be decreased.

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