

IDENTIFICATION AND CHARACTERIZATION OF MICROPLASTICS IN MILK AND WATER SAMPLES AND ANALYZING THE EFFECTS OF TOXICITY IN CELL LINES

Submitted in partial fulfillment of the requirement for the award of
Bachelor of Technology degree in Biotechnology.

By

MAHAVISHNOU R (40230023)

RAMU MITHRAN L (40230040)



**DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF BIO AND CHEMICAL ENGINEERING**

SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

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JEPPIAAR NAGAR, RAJIV GANDHI SALAI, CHENNAI - 600 119

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of MAHAVISHNOU R (40230023), RAMU MITHRAN L (40230040) who have done the project work entitled "IDENTIFICATION AND CHARACTERIZATION OF MICROPLASTICS IN MILK AND WATER SAMPLES AND ANALYZING THE EFFECTS OF TOXICITY IN CELL LINES" under our supervision from September 2023 to April 2024.

Internal Guide

Dr. S. JAYASHREE Ph.D.

External Guide

Dr. KRUPAKAR PARTHASARATHY

Head of the Department

Dr. V. RAMESH KUMAR Ph.D.

Submitted for Viva voce examination held on 25/04/2024

Internal Examiner

(Dr. S. Visha Raveendran)

External Examiner

(Dr. B. DEVI)

DECLARATION

We, **Mahavishnou R (40230023)** and **Ramu Mithran L (40230040)** hereby declare that the Project Report entitled **“Identification and characterization of microplastics in milk and water samples and analyzing the effects of toxicity in cell lines”** done by us under the guidance of **Dr. S. Jayashree Ph.D., (Internal)** and **Dr. Krupakar Parthasarathy (External)** at **Sathyabama Institute of Science and Technology, Chennai, Tamil Nadu, India**, is submitted in partial fulfillment of the requirements for the award of **Bachelor of Technology degree in Biotechnology**.

DATE:

PLACE: Chennai

M. V. Mithran

SIGNATURE OF THE CANDIDATES

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ABSTRACT:

Microplastics are plastic particles that are smaller than 5mm in size and have become a global concern due to their presence in various ecosystems. This study investigates the presence and characterization of microplastics in milk and water samples, as well as their potential effects on human cell lines. A total of 9 samples, comprising 5 water samples and 4 milk samples, were analyzed. Microplastics were initially identified using light microscopy, appearing as long fibers, fragments, and threads. Subsequent characterization through Fourier-transform infrared (FTIR) and Raman spectroscopy revealed the presence of various polymer types, including polyethylene, polyethylene terephthalate, and polyvinyl chloride. Notably, polyethylene was found to be the predominant polymer in the majority of the samples. To assess the potential impact on human health, cytotoxicity assays were conducted using 3T3 cell line. The results demonstrated an increase in cell cytotoxicity with increasing concentrations of microplastics. This study highlights the ubiquitous nature of microplastic contamination in commonly consumed products and underscores the need for further research to elucidate the potential health risks associated with microplastic exposure.

Keywords: Microplastics, Water, Milk, Polyethylene (PE), Cell line study, Cytotoxicity, Human health

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LIST OF ABBREVIATIONS

ACRONYM	EXPANSION
µg/ml	Microgram per millilitre
µl	Microlitre
µm	Micrometre
3T3	Mouse embryonic kidney cell line
A549	Human alveolar epithelial cells
ABS	Acrylonitrile Butadiene Styrene
ANOVA	Analysis of variance
ATR	Attenuated Total Reflectance
Caco-2	Human colorectal adenocarcinoma cells
cm	Centimetre
CO ₂	Carbon dioxide
COPD	Chronic Obstructive Pulmonary Disease
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
FESEM	Field Emission Scanning Electron Microscopy
fibres/day/m ²	Fibres per day per metre square
fibres/m ³	Fibres per metre cube
fibres/mg	Fibres per milligram
FTIR	Fourier Transform Infrared spectroscopy
HEK-2	Human kidney proximal tubular cells
HepG2	Human liver hepatocellular cells
HMC-1	Human mast cells
IC ₅₀	Half maximal inhibitory concentration
IL	Interleukins
LDPE	Low-Density Polyethylene
mg	Milligram
mg/kg	milligrams per kilogram

mg/ml	Milligram per millilitre
ml	Millilitre
mm	Millimetre
MMT	Million metric tonnes
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCM 460	Normal human colon mucosal epithelial cells
nm	Nanometre
particles/L	Particles per litre
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate buffered saline
PE	Polyethylene
PET	Polyethylene terephthalate
pieces/kg	Pieces per kilogram
PO	Programme Outcome
PP	Polypropylene
PPH	Polypropylene Homopolymer
PS	Polystyrene
PSO	Programme Specific Outcome
PVC	Polyvinyl chloride
RAW 264.7	Mouse monocyte <i>cell line</i>
RO	Reverse Osmosis
ROS	Reactive Oxygen Species
rpm	Revolutions per minute
SBR	Styrene Butadiene Rubber
SEM	Scanning Electron Microscopy
SH-SY5Y	Human neuroblastoma cell line
THP-1	Human monocytic cell line
TNF	Tumor Necrosis Factor
U937	Human myeloid leukaemia cell line

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CHAPTER-1

1. INTRODUCTION:

Humans frequently use plastic; the increasing demand for plastic has led to an increase in both the manufacturing and use of plastic materials. Asia contributes over half of the world's plastic manufacturing, which has reached an estimated 8,300 million metric tonnes (MMT) since 1950 (Plastics Europe, 2019). Plastic packaging in the food sector has various advantages, including easier transportation, storage, preservation, and protection against factors such as oxygen, water vapour, and microbes (Kedzierski *et al.*, 2020). Plastics have become widespread pollutants, and as they break down, they turn into microplastics, which are particles smaller than 5 mm in size (Auta *et al.*, 2017). Microplastics comes in two main forms: primary and secondary. Primary microplastics are originally produced as small particles, like resin pellets, used in various products such as cosmetics, toothpaste, soaps, and scrubs (Kim *et al.*, 2018; Liebezeit *et al.*, 2013). On the other hand, secondary microplastics result from the breakdown of larger plastic items into smaller fibres and fragments due to environmental factors like wind, waves, sunlight, and biological processes (Pivokonsky *et al.*, 2018; Kiruba *et al.*, 2022).

Microplastics may enter the body through a variety of products that we use. Microplastics in packaged water bottles come from bottle caps as well as plastic packaging, which can be detected in beverages and plant-based meals (Wright, S.L., *et al.*, 2017). Microplastics are frequently inhaled and ingested by humans, but their potential impact on health remains unknown. Lack of data on exposure to microplastics and danger are critical knowledge gaps that must be addressed to move further (Vethaak & Legler, 2021). Microplastics are now found everywhere in nature, from various habitats worldwide to even the polar regions (Lusher *et al.*, 2015), due to natural processes such as infiltration, river discharge, wind dispersal, ocean currents, and the movement of animals and humans within and between ecosystems. Their persistence and widespread presence pose significant threats to ecosystems, marine organisms, and human health, leading to increasing scientific interest in this form of plastic pollution. In aquatic ecotoxicology, microplastics can potentially serve as carriers for highly toxic pollutants, acting as a source of lipophilic chemicals that accumulate and magnify in the food chain, thereby facilitating the entry of these substances into ecosystems (Hartmann *et al.*, 2017).

Microplastics in the air can cause severe harm to our ecosystems. Microplastics in the atmosphere can come from a wide range of sources, the most prevalent ones being synthetic fabrics, rubber tire breakdown, and urbanization. Microplastics come from a range of sources, like construction materials and textiles, and are spread throughout the air. This dispersion of tiny particles is concerning because people might unknowingly inhale them, which could result in respiratory health problems (Dioses-Salinas *et al.*, 2018). Agricultural activities, such as using sewage sludge as fertilizer and plastic mulching, contribute to the accumulation of microplastics in soil. Microplastics from soil and landfills can also enter freshwater and marine ecosystems, increasing their environmental presence. These particles can be absorbed by food crops grown in such soil, providing a direct route for microplastics to enter the human food chain. Microplastics are also found in poultry feed and environments, leading to their accumulation in chickens. Similarly, farm animals can ingest microplastics, exposing humans to these particles through the consumption of contaminated poultry, meat, and dairy products (Gola *et al.*, 2021).

Microplastics, found widely in different environments, expose humans through several pathways. People can be exposed to microplastics through inhalation, ingestion, and dermal contact, as well as by using various plastic-based products, paint shards, and plastics released into the environment via land, sea, and air. Due to their physical characteristics, microplastics have irregular shapes such as cubic, spherical, and rod shapes, and they can trigger the body to produce toxic effects (Blair *et al.*, 2017). Microplastics are present in significant quantities in indoor dust from places like offices and homes compared to outdoor environments, and people can inhale them. In indoor settings, microplastics originate from various sources, including household items, varnishes, artificial grass used in sports facilities, fibres, and printers. One of the most common sources of microplastics globally is styrene butadiene rubber (SBR), as well as roadway coatings from vehicle emissions and corrosion (Correia Prata, 2018; Dehghani *et al.*, 2017).

Microplastics can release plasticizers and absorb pollutants after being ingested, introducing chemicals and biological toxins into the food web. This can have unpredictable consequences in ecosystems, especially regarding the accumulation and magnification of toxic pollutants within organisms. Microplastics can be released from faeces and then consumed by detritivores such as bacteria

and sediment-dwelling organisms like the lugworm *Arenicola marina*, which can cause bioturbation. There are concerns that microorganisms, including pathogens, could form biofilms on the surface of microplastics (Zettler *et al.*, 2013). A diverse microbial community, known as the 'plastisphere', has been found attached to plastic marine debris in the North Atlantic. This community includes bacteria capable of degrading hydrocarbons, which could affect the fragmentation and degradation of plastic debris. Moreover, specific pathogens, especially a type of *Vibrio* species, have been found on plastic fragments (Carson *et al.*, 2013). Consequently, microplastics can act as carriers for waterborne human pathogens, affecting the cleanliness and quality of water.

CHAPTER-2

2. REVIEW OF LITERATURE:

2.1 MICROPLASTICS CYCLE:

Microplastics have been identified in every part of the environment (Dris *et al.*, 2016), emphasizing the importance of understanding how they contaminate our food and drink to evaluate the extent of this contamination. The sources of microplastic particles include activities related to fishing, industry, home, and agriculture, as well as the use, manufacturing, and disposal of plastic items. This highlights the main ways microplastics can enter the environment, especially through water and air, before potentially making their way into the food chain. This intricate web of interactions helps to explain how microplastics might spread throughout the environment and affect food and beverages. Indeed, the food chain involves many steps, including processing, treating, and distributing food, each of which could add microplastics to the process. However, efforts to understand how the human food chain might be contaminated consider several pathways, as discussed in the literature, including seawater (Buchanan *et al.*, 1971), wastewater, soil, sludge (Zubris *et al.*, 2005), and both surface and groundwater contamination (Eriksen *et al.*, 2013). Lakes and rivers, key sources of drinking water after treatment and purification, have been confirmed to be contaminated with microplastics, a well-documented problem (Faure *et al.*, 2012).

Airborne microplastics represent another route through which these particles can enter the human diet. Studies have found that indoor and outdoor air contains textile fibres, with microplastics constituting 33% of these. Outdoor air typically has 0.3 to 1.5 microplastic fibres/m³, but indoor air can contain significantly more, from 1.0 to 60.0 fibres/m³. Concerning human health, a study discovered that 87% of human lung samples contained fibres from both plants and plastics. Smaller fibres, which are more likely to be inhaled, can penetrate deep into the lungs and impact respiratory health (Correia Prata, 2018), whereas larger fibres tend to settle as dust. In indoor settings, these larger fibres can deposit at rates ranging from 1,586 to 11,130 fibres/day/m², equivalent to 190 to 670 fibres/mg of settled dust. Areas with high foot traffic, like city streets and industrial areas, pose a higher risk of airborne microplastic exposure (Dehghani *et al.*, 2017), and the shedding of fibres from

textiles is a significant source of concern (Carr, 2017). Therefore, it's possible to ingest microplastics not only by consuming contaminated food or through contact with airborne dust on food but also through daily activities in environments where these particles are present (Dris *et al.*, 2017).

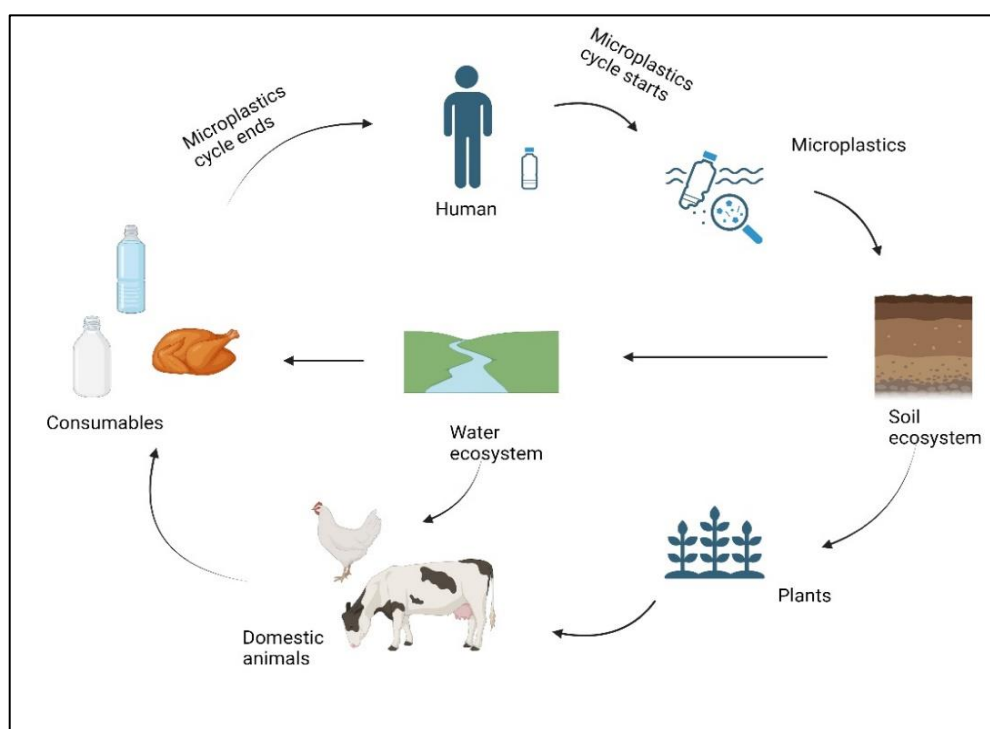


Fig 2.1: Microplastics cycle in environment

2.2 MICROPLASTICS IN SOIL:

Microplastics infiltrate soil through several channels, including compost and sewage sludge application, plastic mulching, irrigation, littering, and atmospheric deposition. Inadequate waste management has notably led to the accumulation of plastic mulch remnants in soil, which degrade over time due to factors like physical wear and UV radiation, thus contributing to the microplastic load (Bläsing, M., & Amelung, W., 2018). Once embedded in soil, microplastics elicit a spectrum of detrimental effects on soil ecosystems, altering physical characteristics, diminishing fertility, and disrupting microbial communities. Furthermore, the ingestion of microplastics by earthworms facilitates their transfer up the food chain, posing risks to terrestrial organisms and potentially human health.

Microplastics in soil also serve as vectors for contaminants, impacting soil biota and soil ecological dynamics (Wang, C., Zhao, J., & Xing, B., 2020). Soil organisms' movements aid in microplastic redistribution within soil layers, with

agricultural and forested soils exhibiting higher plastic absorption compared to urban settings. Soil samples have previously been obtained at various depths ranging from 0–10 cm to 0–30 cm, often within the ploughing layer in agricultural regions. The highest concentration of MPs, 236,000 pieces per kilogram (pieces/kg), was found in cropland in Northern Jutland, Denmark. This high percentage was linked to the use of sewage as a fertilizer in agriculture (Vollertsen, J., & Hansen, A.A., 2017). The largest mass concentration of microplastics was found in industrial soil in Australia, at 67,500 milligrams per kilogram (mg/kg). The deposition of both treated and untreated industrial waste into the soil was attributed to the higher concentration.

The soil ecosystem plays a pivotal role in the microplastic lifecycle, where plastics gradually fragment into micro- and nano-sized particles, exhibiting remarkable durability over extended periods. This cycle begins with human disposal of plastics into soil environments, where microplastics, typically smaller than 5 mm, are readily identified. As plants draw nutrients, they inadvertently intake microplastics from the soil, initiating their entry into the food chain. Given that plants form the foundation of many food webs, herbivores are particularly susceptible to microplastic ingestion, potentially leading to microplastic accumulation within their bodies. This intricate process underscores the pervasive reach of microplastics throughout ecosystems, posing a concern for organisms across trophic levels, including humans.

2.3 MICROPLASTICS IN PLANTS:

The accumulation of tiny plastic particles, known as microplastics, in soil has raised concerns about their potential impact on plant growth and development. These microplastics often originate from degraded plastic waste or synthetic textile fibres and can interfere with soil properties and plant physiology. When present in soil, they may obstruct water and nutrient absorption by plant roots, consequently affecting plant growth and productivity. Furthermore, these particles have the potential to enter plant tissues, although the extent and consequences of this uptake are still being investigated. Understanding the interaction between microplastics and plants is crucial for mitigating any adverse effects on agricultural productivity and

environmental sustainability (Watteau, F., Dignac, M.-F., Bouchard, A., Revallier, A., & Houot, S., 2018).

Several studies have investigated the direct effects of microplastics on plants, focusing particularly on root growth and seed germination. Larger microplastic particles, ranging from 100 nm to 5 mm in size, can indirectly affect plants by altering and potentially disrupting soil structure. Smaller particles, on the other hand, can directly obstruct seed pores, leading to a potential slowdown or complete inhibition of water absorption. One study found that an 8-hour exposure to plastic fragments of sizes 50, 500, and 4750 nm at concentrations of 10³ to 10⁷ particles per millilitre reduced seed germination by 78%, attributing this decrease to the physical blocking of seed pores by microplastics. The researchers used fluorescent dyes and confocal microscopy to demonstrate this effect and discovered microplastic buildup on root hairs at a later stage (Bosker, T., Bouwman, L.J., Brun, N.R., Behrens, P., & Vijver, M.G., 2019). Another study visualized the accumulation of 100 nm microplastics in the roots of broad beans (*Vicia faba*) using laser confocal scanning microscopy (Jiang *et al.*, 2019). In this experiment, microplastics may have impeded cell wall pores or cell connections responsible for water and nutrient movement within the plant, potentially explaining the observed increase in antioxidant enzyme activity and decreased plant development caused by microplastic buildup (Li *et al.*, 2020).

Researchers have also demonstrated the localization of fluorescently tagged microplastics within various tissues of wheat and lettuce plants. Using confocal laser scanning microscopy, they observed polystyrene microplastic microbeads in the root, shoot, and leaves of wheat plants (Li *et al.*, 2019). In lettuce, fluorescent polystyrene microbeads (0.2 μ m) were found trapped extracellularly in the root cap, visible to the naked eye as a dark green tip. Confocal microscopy revealed their presence in the cell wall tissue of the root and vascular system, indicating the passage of microbeads through intercellular gaps via apoplastic transport. Following the transpiration stream, these microbeads travelled through the vascular system, forming chains and bunch-like formations in various locations but being evenly distributed throughout the leaf. However, it's important to note that these investigations subjected plants to microplastic particles in aqueous solutions or hydroponic systems rather than in actual soil conditions. If these crop varieties were grown in soil contaminated with microplastics, the results could be influenced by

factors such as the type and concentration of microplastics, plant species, soil type, and experimental settings (Chae & An *et al.*, 2020). In a notable study, mung bean (*Vigna radiata*) plants were directly exposed to soil contaminated with nanoplastics, and the results were significant. They observed an 83.3% reduction in root growth when the concentration of nanoplastics in the soil was 100 mg/kg. Additionally, nanoplastic particles were found to accumulate in the leaves of the mung bean plant. The contaminated mung bean plant had a detrimental impact on the growth rate of the African giant snail that consumed it, highlighting the potential for microplastics to enter the food chain through plant-based diets. This underscores the critical importance of gaining a comprehensive understanding of the *in vivo* effects of microplastics on a wide range of food crop plants, especially those with edible roots, to assess the potential impact on food safety and security.

2.4 MICROPLASTICS IN POULTRY:

The introduction of microplastics into ecosystems can lead to their accumulation and distribution across food chains, potentially harming various species within these ecosystems (Ross *et al.*, 2021). When ingested, microplastics may result in toxic effects that are particularly worrying regarding the quality of chicken, an important dietary source for humans. Research has shown that microplastics can cause a range of negative health effects in organisms, such as behavioural changes, decreased survival and reproduction rates, reduced movement, smaller body sizes, increased inflammation (including damage to the intestines), neurological harm, oxidative stress, DNA damage, and disruptions in lipid and energy metabolism. These effects highlight the serious risks posed by microplastics, though it's worth noting that these findings often come from laboratory settings with high microplastic concentrations (Crump *et al.*, 2020; Prüst *et al.*, 2020). However, the evidence points to potential dangers if microplastic levels rise in natural food sources and among wildlife, emphasizing the urgency of monitoring and reducing microplastic pollution to protect living organisms and ecosystems.

Birds, due to their sensitivity to environmental changes, serve as key indicators for studying the effects of pollution. Many species, both terrestrial and aquatic, have been observed accumulating plastic waste, posing significant toxicity risks. Terrestrial birds, integral to the food web and ecosystem dynamics, have been

documented to have microplastics in their digestive systems across a wide range of species (Carlin *et al.*, 2020). Research indicates that raptors, too, are not exempt from macro and microplastic ingestion. Aquatic birds, reliant on marine life for food, often ingest prey that has accumulated microplastics, leading to widespread plastic consumption among these species (Barbieri *et al.*, 2010). Studies suggest a dramatic increase in the number of seabird species ingesting plastics since the 1960s, projecting that by 2050, over 99% of aquatic bird species will have ingested plastic. This underscores the pervasive nature of microplastic pollution and its potential impacts on bird populations.

A recent investigation has uncovered microplastic particles within the digestive parts of chickens, notably in the gizzard and crop. The study found a predominance of relatively large particles, between 300 and 500 µm, over smaller sizes. These particles mostly appeared as fragments and were identified in various colours, with yellow being predominant in the gizzard and red in the crop. Chemical analyses revealed the presence of several polymer types, including polyvinyl chloride (PVC), polystyrene (PS), low-density polyethylene (LDPE), and polypropylene homopolymer (PPH), with LDPE and PVC being the most common.

This finding raises important health concerns for humans due to the presence of microplastics in chickens, a common food source. The primary avenues for these chickens to ingest microplastics likely include their feed and the environment of the farms where they are raised. Future research should aim to thoroughly investigate the sources of microplastic exposure in chickens and examine how these particles might transfer and accumulate within the food chain. Understanding the full implications of microplastic pollution on food safety is crucial for addressing this emerging threat.

2.5 MICROPLASTICS IN WATER:

Although a significant amount of research has been done on the issue of microplastic pollution in coastal ecosystems, freshwater and estuary environments have received less attention. For efficient pollution control, it is essential to comprehend the origins and migration of microplastics from land to water. Microplastics are a byproduct of human activity and can be found in soil due to landfill leachate, unmanaged trash disposal, and agricultural mulching (Dioses-Salinas *et*

al., 2018). Since untreated sludge is frequently used on farms as fertilizer, it instantly introduces microplastics into farms and influences the ecosystem. Larger plastic objects are the main carriers of microplastics; these things are broken down by wind, rain, sunlight, and water movement and finally end up in water bodies like rivers, ponds, and reservoirs. Areas near urban centres or with high population densities tend to have more microplastics (Tang *et al.*, 2021), indicating a close relationship between human activities and the presence of microplastics in aquatic ecosystems.

Numerous studies have highlighted the harmful effects of microplastics on freshwater, marine, and ocean fish species. A study examined microplastic consumption in two types of freshwater streams: urban and non-urban. Fish samples from the urbanised freshwater stream showed a significant presence of microplastics, with larger systems surrounding the river basin being the primary source. Over 100 different fish species were found to have ingested microplastics, with fibres and hard fragments found in stomach cells. Additionally, fish samples from areas near urban centres showed higher levels of microplastic ingestion, along with other additives such as chemicals, heavy metals, and dyes (Gola *et al.*, 2021).

Studies indicate that tap water contributes significantly to microplastic ingestion, with >88% of synthetic particles ingested by humans coming from tap water. Recent research has detected microplastics in drinking water globally, with 93% of bottled water samples found to be contaminated. The maximum reported abundance of microplastics in tap water was 628 particles/L, while contamination levels in bottled water were nearly 8 times higher. Surprisingly, water sold in glass bottles had higher microplastic counts than single-use PET bottles. However, some regions, like China, reported lower microplastic levels in bottled water, with 2–23 items per bottle. The high statistical heterogeneity in these limited data sets has hindered meta-analyses and the progress of human health risk assessments related to microplastics in drinking water. Addressing these data limitations is crucial to advancing risk assessment efforts associated with microplastics in drinking water (Li *et al.*, 2023).

2.6 MICROPLASTICS IN MILK:

Milk consumption is crucial for human nutrition throughout life, highlighting the importance of ensuring its food safety, especially regarding microplastic

contamination. Despite this, the development of microplastics in milk during production, from farms to dairy processing, has not been thoroughly studied. Given the potential risks associated with microplastics, there is a growing emphasis on the need to monitor and conduct risk assessments of microplastic levels in food products, as food-based exposure to microplastics is increasing. For instance, Turkey produced approximately 10 million metric tonnes of cow's milk in 2022 (Başaran *et al.*, 2023).

Milk and dairy products are essential for human nutrition and are a significant global commodity, with dairy product exports reaching 75 million metric tonnes and global milk output estimated at 843 million metric tonnes in 2018. The awareness of the link between diet and health has led to the production of various milk varieties, including low-fat, low-calorie, fortified, skimmed (fat-free or non-fat), and lactose-free milks. However, despite the focus on hygiene and pathogen testing in milk production, the potential contamination of milk with microplastics from various sources, such as poor equipment cleanliness, the environment, water supply, and plastic-based packaging materials, has not been thoroughly studied. There is a lack of research on the occurrence and abundance of microplastics in milk, raising concerns about their potential health impacts on consumers. It is crucial to address these research gaps to ensure the hygiene and safety of milk products (Kutralam-Muniasamy *et al.*, 2020).

2.7 TOXICITY IN HUMANS:

Researchers are increasingly concerned about the potential impact of microplastics on human health and small vertebrates, as evidenced by instances where these particles have been found within species, leading to detrimental effects. While studies indicate that microplastics can readily infiltrate various parts of the body, severe adverse effects are relatively uncommon. In simpler terms, while microplastics can reach different bodily regions, serious health issues due to their presence are not widespread. Microplastics have the potential to affect a range of bodily functions, including the immune, respiratory, endocrine, reproductive, and digestive systems. For example, ingestion of microplastics can induce inflammation in the gastrointestinal tract and irritation in the digestive system (Bouwmeester *et al.*, 2015). While several studies have suggested possible illnesses linked to suspected

microplastics, further research is necessary to confirm these findings. Additionally, the use of polypropylene (PP) particles has demonstrated various harmful effects on different cell lines, depending on the particle size (approximately 20 μm and 25–200 μm) and concentrations used in tests. This suggests that the interaction of microplastics with humans can lead to cytotoxicity, hypersensitivity, undesirable immune responses, and acute effects like hemolysis, potentially posing a risk to human health (Hwang *et al.*, 2019).

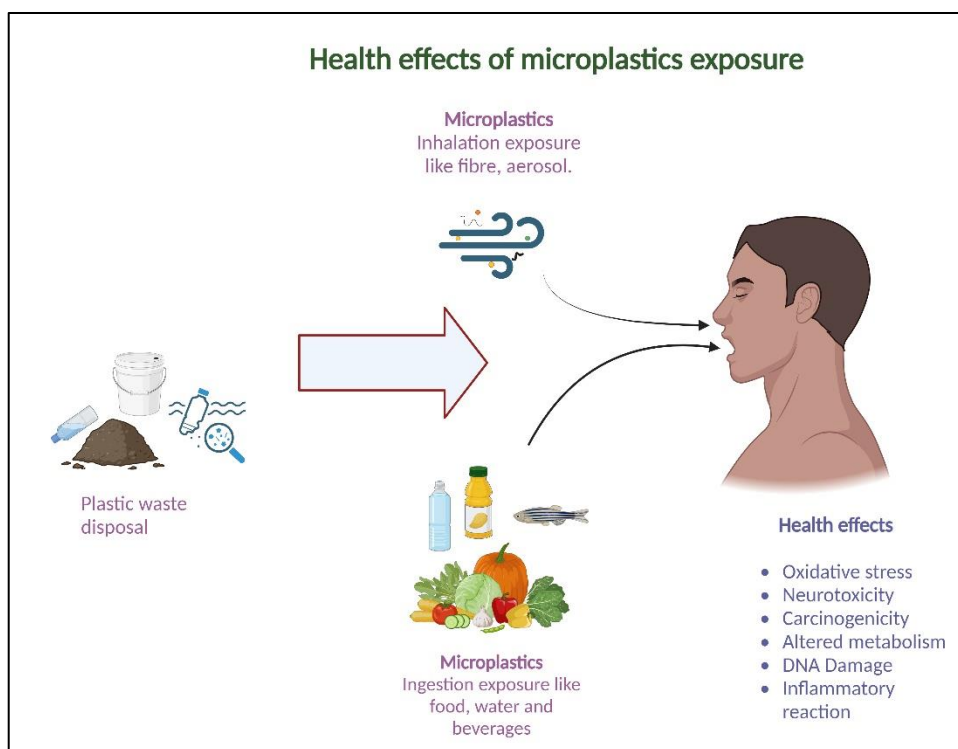


Fig 2.2: Effects of microplastics in humans

Microplastics pose various health risks for humans. Even at low doses, polystyrene microplastics can cause cytotoxic and inflammatory effects in the lungs, potentially increasing the risk of chronic obstructive pulmonary disease (COPD) at high concentrations (Bouwmeester *et al.*, 2015). These particles can disrupt the immune system, potentially leading to autoimmune diseases, as seen in studies showing reduced activation of dendritic cells, inhibition of T-helper type 2 responses, and increased production of IL-10 in mice (Saravia *et al.*, 2014). Microplastics can also cause genotoxic effects, potentially leading to cancer, as evidenced by the concentration of DNA damage observed in cells exposed to polystyrene nanoparticles (Fadeel, Pietroiusti, & Shvedova, 2012). In terms of neurological impacts, small polystyrene nanospheres have been found to be toxic to neural cells,

and microplastics can induce neurotoxicity through oxidative stress and brain microglia activation (Murali *et al.*, 2015). Furthermore, microplastics can enter the gastrointestinal tract, affecting the gut microbiome and causing dysbiosis, decreased resistance, pathogen outbreaks, and metabolic dysfunction.

2.8 MICROPLASTICS EFFECTS IN HUMAN CELL LINES:

Various human cell lines have been utilized to investigate the effects of microplastics on cellular processes. Caco-2 cells, representing intestinal epithelial cells, exhibited minimal cytotoxicity to polystyrene microplastics. However, at larger dosages, these microplastics caused oxidative stress, mitochondrial dysfunction, and reduced nutrient absorption. Similarly, NCM 460 cells (normal human colon mucosal epithelial cells) showed little acute toxicity with polystyrene microplastics but disrupted the redox equilibrium at higher concentrations. A549 cells (human alveolar epithelial cells) displayed lower proliferation and morphological alterations in response to polystyrene microplastic exposure, suggesting potential harm from inhaled particles. Mast cells released inflammatory cytokines like IL-6 in response to polypropylene microplastics, and macrophages from the U937 cell line produced cytokines, indicating an inflammatory response to microplastics. THP-1 cells (representative of dendritic cells) exposed to microplastics generate inflammatory cytokines such as IL-6.

Organ-specific cell lines have also been studied. HepG2 cells (human liver hepatocellular cells) exposed to polyethylene microplastics displayed altered lipid metabolism, while polystyrene microplastics increased oxidative stress, inflammatory markers, and mitochondrial damage in HEK-2 cells (human kidney proximal tubular cells). Human neuroblastoma-like SH-SY5Y cells, when exposed to polystyrene nanoplastics, showed signs of oxidative stress, dysfunctional mitochondria, and apoptotic cell death pathways (Barceló, Picó, and Alfarhan, 2023). Studies employing mouse models have suggested potential impacts of microplastics on cellular toxicity and gut microbiota. Animal studies have revealed that magnetic nanoparticles can lead to reproductive toxicity and affect offspring health. In mice, high-level exposure to polystyrene particles resulted in ovarian inflammation, reduced oocyte quality, testicular inflammation, reduced sperm quality, and harm to the blood-testis barrier (Feng *et al.*, 2023). Factors like particle

size, polymer type, shape, charge, concentration, and exposure pathways influence the outcome of microplastic exposure, with potential side effects including oxidative stress, inflammation, metabolic disturbances causing gastrointestinal issues, liver damage, reproductive problems, and neurotoxic consequences (Setälä, Fleming-Lehtinen, and Lehtiniemi, 2014).

One study found that polystyrene particles can induce phagocyte toxicity, with larger nanoparticles generating a quantifiable respiratory burst in monocytes and promoting the release of cytokines such as IL-6 and IL-8 from macrophages and monocytes (Desforges, Galbraith, and Ross, 2015). In Caco-2 cells, both 0.1 μm and 5 μm polystyrene microplastic particles showed minimal impact on cell viability and oxidative stress, but the larger 5 μm particles resulted in more significant mitochondrial depolarization compared to the smaller 0.1 μm (Hwang *et al.*, 2020). Another study investigated the effect of polystyrene microplastics on mouse and human kidney cells, finding increased amounts of misfolded proteins and mitochondrial reactive oxygen species (ROS) at different dosages. Furthermore, the cells exhibited inflammation and increased endoplasmic reticulum stress after exposure to polystyrene microplastics. Polyethylene microplastics have been found to be responsible for genomic instability in human peripheral lymphocytes, and chronic exposure to microplastics may contribute to human genomic instability, potentially resulting in diseases like cancer, implying a serious threat to human health (Nan *et al.*, 2020). Recent in vitro studies have shown that microplastics can affect human cells, causing issues ranging from inflammation to DNA damage. One study examined polypropylene particles and found low cytotoxicity at different sizes and concentrations but stimulation of the immune system at high concentrations of small-sized particles, potentially leading to hypersensitivity through increased cytokines and histamines in peripheral blood mononuclear cells (PBMCs), RAW 264.7, and HMC-1 cells (Dris *et al.*, 2016). Another study investigated the impact of size, concentration, and particle shape of PVC and acrylonitrile butadiene styrene (ABS) microplastics on cytotoxicity in various cell types. Larger PVC particles induced the release of IL-6 and TNF- α , while smaller ABS particles triggered IL-6 at high concentrations and TNF- α at all concentrations, suggesting that microplastics can stimulate immune responses (Hwang *et al.*, 2020; Han *et al.*, 2020).

Magnetic nanoparticles may pose hazards to the liver, as they can accumulate in the livers of marine animals and result in cytotoxicity. Research using the HepG2 cell line (an in vitro model of the human liver) has examined the impact of magnetic nanoparticles. One study found that certain polystyrene magnetic nanoparticles with surface functionalization were more hazardous to HepG2 cells than unmodified magnetic nanoparticles. Another study demonstrated that exposure to spherical polyethylene magnetic nanoparticles caused dose-dependent cell membrane damage. Inhaled magnetic nanoparticles can also pose risks to the human respiratory system. Studies using the A549 cell line (representing human lung epithelial cells) have explored the effects of magnetic nanoparticles. Exposure to different sizes of polystyrene magnetic nanoparticles led to reduced cell viability, cell cycle arrest, inflammation, and altered protein expression. Other studies exposed human alveolar A549 cells to polystyrene magnetic nanoparticles, resulting in reduced cell proliferation, morphological changes, and magnetic nanoparticle uptake, suggesting toxic consequences. Additionally, research found that healthy respiratory cells can adapt to low levels of repeated magnetic nanoparticle exposure (Nan *et al.*, 2020).

CHAPTER-3

3. AIM AND SCOPE OF PRESENT INVESTIGATION:

3.1 AIM:

The aim of the study is to employ Fourier Transform Infrared (FTIR) spectroscopy and Raman spectroscopy, for the identification and characterization of microplastics in milk and water samples. Additionally, this research aims to analyse the potential toxic effects of microplastics on cell lines, providing insights into the health risks associated with microplastic contamination in daily consumables.

3.2 SCOPE:

1. Sample Collection and Preparation:

- Systematically collect milk and water samples from various sources to ensure a comprehensive analysis.
- Prepare samples for spectroscopic and imaging analyses, involving processes such as filtration and drying, to identify microplastics from the liquids using light microscopy.

2. To Identify and Characterize Microplastics:

- Utilize FTIR spectroscopy to identify the chemical composition of microplastics present in milk and water samples.
- Employ Raman spectroscopy for further characterization of microplastics, focusing on their molecular and structural information.

3. Data Analysis and Microplastics Profiling:

- Analyse spectroscopic and imaging data to profile the types of microplastics detected in the samples, including polymer type, size distribution, and concentration.
- Compare the findings from FTIR and Raman spectroscopy to validate the identification and characterization of microplastics.

4. Toxicity Assessment:

- Investigate the cytotoxic effects of identified microplastics on selected cell lines using appropriate in vitro assays.

5. Assessment of Microplastic Potential Health Risks:

- Integrate the data obtained from the characterization of microplastics and their toxicity profiles to assess potential health risks to humans.
- Discuss the implications of microplastic contamination in milk and water on human health, considering both direct ingestion and potential bioaccumulation effects.

6. Contribution to Knowledge and Policy Development:

- Contribute to the scientific understanding of microplastic pollution in consumable liquids and its implications for human health.
- Provide data that could inform policymakers and stakeholders about the urgency and importance of addressing microplastic pollution.

CHAPTER-4

4. MATERIALS AND METHODS:

4.1 WATER SAMPLES:

4.1.1 SAMPLE COLLECTION:

In this investigation, the focus lies on assessing the occurrence and characteristics of microplastics within a range of water sources, including tap water, distilled water, Milli-Q water, reverse osmosis (RO) water, and commercially available bottled water. The methodology employed involves filtration and microscopic analysis to determine the levels of microplastic contamination and to compare the composition of microplastics found in these various water sources. Understanding the distribution and presence of microplastics is fundamental for formulating strategies to mitigate their impact and for enhancing practices in water resource management.

Table 4.1: Types of water samples collected

Sl. No.	Type of sample
1.	500 ml- Tap water
2.	500 ml- Distilled water
3.	500 ml- Milli Q water
4.	500 ml- RO water
5.	500 ml- Packaged drinking water

Samples were procured from five distinct water sources for the purpose of identifying and analysing microplastic particles present in them. These sources encompass tap water, distilled water, Milli-Q water (noted for its high purity), RO-treated water, and bottled water. The objective was to ascertain whether these widely used water varieties harbour microscopic plastic particles, known as microplastics. To identify microplastics from the water samples, a filtration process was utilized, employing Whatman filters (Grade 1, CAT No. 1440–125) in conjunction with the conical flask for the filtration task. Post-filtration, the microplastics trapped filters were collected onto petri dishes for further examination.

Extreme caution was exercised throughout the process to prevent any external contamination of the samples. All equipment, including conical flasks, beakers, funnels, and petri plates, was thoroughly sterilized before use to ensure purity.

4.1.2 FILTRATION METHOD:

The filtration operation was performed within a laminar airflow environment to safeguard against contamination from airborne microplastics, thus maintaining the integrity of the water samples. A Whatman filter paper (Grade 1, CAT No. 1440–125) was selected for the filtration, allowing for the gradual passage of the water sample through the filtration apparatus. The duration of this process varied, typically around 30 minutes, depending on the specific characteristics and volume of the water sample being filtered. Following this step, the filter paper was left to dry at room temperature.

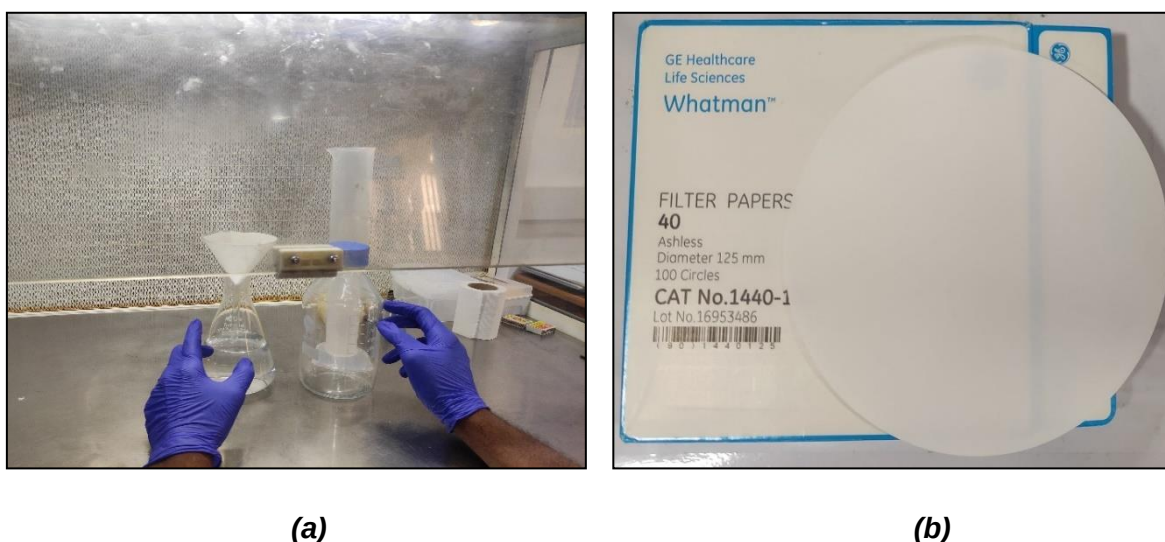


Fig 4.1: (a) Filtration setup of water samples in laminar hood (b) Whatman filter paper

Once dry, the filter paper was subjected to microscopic examination, enabling the identification and analysis of the captured microplastic particles. This approach was integral to quantifying the prevalence of microplastics in the sampled water sources and provided a comparative understanding of microplastic composition across different types of water. The findings from this meticulous investigation are anticipated to contribute valuable insights toward developing effective approaches for the mitigation of microplastic pollution and promoting sustainable water management practices.

4.2 MILK SAMPLES:

4.2.1 SAMPLE COLLECTION:

The selection of milk products for analysis was guided by their popularity and widespread availability in both local and national markets. Consequently, four distinct milk samples were chosen: two packaged milk samples, one milk sample in a tetra Pak carton box, and one organic milk sample obtained directly from a cow. These samples were procured from various retail outlets and supermarkets across Chennai, predominantly available as pasteurized milk packaged in low-density polyethylene bags, a common packaging choice for household and coffee milk consumption. Prior to the microplastic extraction process, the milk samples were stored under controlled conditions to maintain their integrity.

Table 4.2: Types of milk samples collected

Sl. No.	Sample name	Type of sample
1.	M1	500 ml- Packaged
2.	M2	
3.	M3	500 ml- Tetra Pak
4.	M4	500 ml- Organic milk

4.2.2 FILTRATION METHOD:

This investigation employed a refined adaptation of the standard filtration technique, specifically tailored for the identification of microplastics from milk samples in a laboratory environment. The initial preparation involved homogenizing the milk by shaking, followed by the transfer of the liquid into a sterilized conical flask. Recognizing the potential obstruction caused by milk's high fat and casein content during filtration, especially when cold, the samples were gently warmed to reduce viscosity and facilitate the passage of fat clusters and globules through the filter.

The methodological approach commenced with measuring the required volume of milk in a glass flask and transferring it into a beaker for processing. All relevant laboratory apparatus, including conical flasks, spatulas, beakers, funnels, and petri dishes, were thoroughly sterilized before use to prevent any contamination.

The entire experimental procedure was conducted within a laminar airflow cabinet to prevent airborne microplastic contamination of the milk samples.

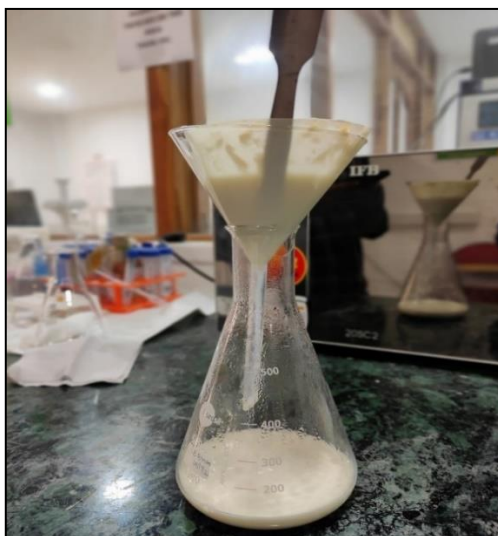


Fig 4.2: Filtration setup of milk samples

After the preliminary boiling of the milk for approximately 30 minutes, the samples were gradually filtered through the setup. In this modified protocol, Whatman filter paper (Grade 1, CAT No.1440-125) with a pore size of 11 μm was utilized. The filtration of milk was handled carefully to circumvent potential blockage of the filtration apparatus by solid milk constituents. Despite these precautions, filters occasionally became clogged during the processing of high-fat milk samples, necessitating the use of fresh filters to complete the procedure. For each milk sample, triplicate filter specimens were prepared and, with the aid of metal forceps, transferred to individual petri dishes for subsequent drying in an incubator and reserved for analysis. The duration of filtration varied according to the specific properties and volume of each milk sample. After filtration, the filter papers were left to dry at room temperature for 24 hours.

4.3 IDENTIFICATION USING LIGHT MICROSCOPY:

The identification of microplastics in the filter papers was carried out using a light microscope with 4X and 10X magnification lenses. The dried filters were meticulously examined under the microscope (Masura, Baker, Foster, and Arthur, 2015). According to these criteria, an object was considered microplastic if, in contrast to biological materials, it distorted or maintained its shape when subjected to pressure from metal tweezers without breaking apart. During the microscopic

examination, the potential microplastic particles were scrutinized to distinguish them from other non-plastic materials based on their physical characteristics. The identified microplastics were then counted and categorized according to their colour and shape, providing an overview of the diversity of microplastic particles present in the milk samples.

To further document the properties of the confirmed microplastic particles, microscope photographs were captured. These visual records served as evidence of the microplastics' presence and aided in the subsequent analysis and characterization of their morphological features, such as size, colour, and shape.

The microscopic examination, coupled with the categorization and visual documentation, provided valuable data for further analysis and contributed to a comprehensive understanding of the microplastic contamination in the water and milk samples under investigation.

4.4 CHARACTERIZATION:

4.4.1 RAMAN SPECTROSCOPY:

Raman spectroscopy, in conjunction with confocal imaging, is a powerful analytical technique employed for the chemical characterization of microplastics identified from filter papers. This technique provides a non-destructive and highly specific method for identifying the polymer composition of microplastic particles.

For analysis, Raman spectroscopy (RENISHAW InVia Confocal Microscope, United Kingdom) was used, a near-infrared laser (785 nm) was utilized to excite the molecular vibrations within the microplastic particles, generating inelastic scattering of light known as the Raman effect. Prior to sample analysis, a background scan was performed to account for any potential interference or background signals. To ensure accurate and comprehensive spectral acquisition, each microplastic specimen was analysed by collecting the Raman spectrum over a wide range, typically from 200 to 3700 cm^{-1} . This spectral range encompasses the characteristic vibrational modes of various polymer types, enabling the detection of their unique spectral fingerprints.

To obtain high-quality spectra and minimize potential laser-induced damage, the laser power, acquisition duration, and number of accumulations were carefully

optimized based on the colour and properties of the microplastic particles under investigation. Typically, the final spectrum for each sample was assembled by averaging three consecutive scans, enhancing the signal-to-noise ratio and improving the reliability of the acquired data. The obtained Raman spectra were then compared against a comprehensive library of reference spectra for known plastic polymers. This comparison involved evaluating the positions and relative intensities of the characteristic peaks in the acquired spectra and matching them with the reference spectra. High-scoring matches with consistent peak positions and intensities were identified as the most probable polymer compositions of the microplastic particles.

Raman spectroscopy offers several advantages in the characterization of microplastics from filter papers. It provides high spatial resolution, allowing for the analysis of individual microplastic particles, even within complex mixtures or matrices. Additionally, the technique is non-destructive, preserving the integrity of the samples for potential further analyses. The integration of Raman spectroscopy into the characterization workflow for microplastics identified from filter papers enables a comprehensive understanding of their chemical nature, contributing to a better assessment of their environmental implications and potential mitigation strategies.



Fig 4.3: Filtered samples in petri plates for characterization

4.4.2 FTIR:

Fourier Transform Infrared (FTIR) spectroscopy is a powerful analytical technique widely employed for the characterization and identification of microplastics recovered from various samples, including those identified from filter papers. This technique offers a rapid and accurate means of determining the polymer type of microplastic particles larger than 10 micrometres.

The characterization process using FTIR spectroscopy was analysed using Jasco FTIR 6600 (Japan), involves comparing the obtained spectra of the suspected microplastic particles with reference spectra of known plastic polymers. This comparison enables the identification of the specific polymer type by matching the characteristic absorption bands corresponding to the functional groups present in the polymer structure. In the case of microplastics identified from water and milk samples using filter papers, the particles were first carefully transferred onto an Attenuated Total Reflectance (ATR) crystal or substrate compatible with the FTIR instrument. The ATR accessory allows for the direct analysis of solid or semi-solid samples without extensive sample preparation.

During the FTIR analysis, the microplastic particles were exposed to infrared radiation, which interacted with the molecular bonds within the polymer structure. The resulting absorption or transmission spectrum, which is a unique fingerprint of the material, was recorded and compared against a library of reference spectra for various plastic polymers. By meticulously analysing and interpreting the characteristic peaks and bands in the obtained spectra, it became possible to identify the chemical composition and polymer type of the suspected microplastic particles. Furthermore, the FTIR analysis provided valuable information about the structural and chemical properties of the identified microplastics. This information can aid in understanding the potential sources, degradation patterns, and environmental implications of these microplastic contaminants.

The combination of microscopic examination and FTIR spectroscopy offers a comprehensive approach to characterizing microplastics identified from filter papers, enabling accurate identification of polymer types and gaining insights into their chemical nature and potential environmental impacts.

4.5 CELL CULTURE:

3T3 cell line were used for the experiment. These cells were cultured in T25 flasks filled with DMEM medium, enriched with 10% Fetal bovine serum (FBS) and a 1% mix of antibiotics (penicillin at 100 $\mu\text{g}/\text{ml}$ and streptomycin at 100 $\mu\text{g}/\text{ml}$). The culture environment was kept humidified with a 5% CO_2 atmosphere at 37°C. Sub-culturing was performed when cells reached a confluency of 70%–80%. At this stage, old media was removed, and cells were cleansed twice with 1 ml of sterile 1X PBS to eliminate any debris and external contaminants. Following this, 1 ml of trypsin-EDTA was applied to release the cells adhered to the flask surface, and the mixture was incubated under 5% CO_2 at 37°C for 3 minutes, ensuring cell detachment. To halt the trypsin's activity, 1 ml of DMEM supplemented with 10% FBS was introduced to the flask.

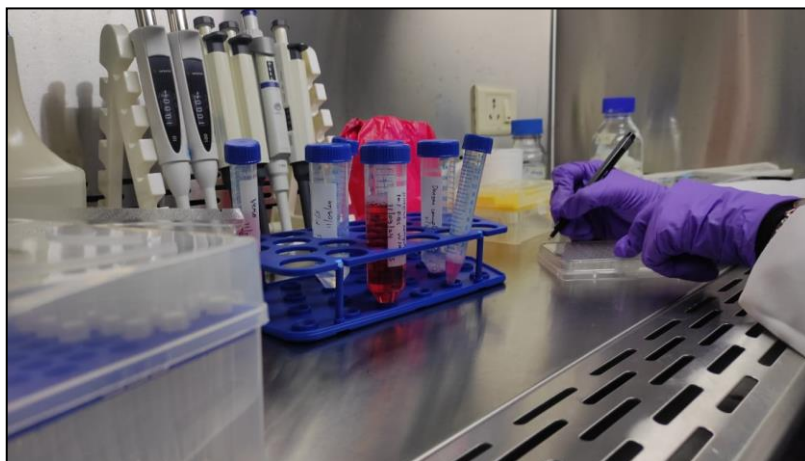


Fig 4.4: Culturing of 3T3 cells



Fig 4.5: 3T3 cells cultured in T25 flask

This mixture was then transferred to a 15-ml centrifuge tube and spun at 1200 rpm for 10 minutes. Post-centrifugation, the supernatant was disposed of, and the cell pellet was resuspended in DMEM enriched with 10% FBS. Cell concentration was subsequently determined using a haemocytometer in preparation for further experiments.

Table 4.3: List of cell lines

Sl. No.	Type of cell line	Cell line	Medium used
1.	Mouse embryonic fibroblast cell line	3T3	DMEM with 10% FBS

4.6 EVALUATION OF CYTOTOXICITY OF MICROPLASTICS IN CELL LINES:

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is a widely used colorimetric technique for evaluating the cytotoxicity and viability of cells exposed to various substances, including microplastics. This assay provides a quantitative measure of metabolically active cells by assessing their ability to reduce the yellow tetrazolium salt to insoluble purple formazan crystals.



Fig 4.6: Microplastics + 0.01% Tween 20 solution

In the experimental setup, a specific number of cells (approximately 10,000) were cultured in each well of a 96-well plate. The adherent cells were allowed to attach to the surface of the wells by incubating them under standard conditions (37°C, 5% CO₂) for a predetermined period. The microplastic samples were

prepared by scraping polyethylene terephthalate (PET) from plastic bottles, which served as a representative microplastic material. Approximately 30 mg of PET was suspended in a 0.01% Tween 20 solution at a concentration of 10 mg/ml. This suspension was subjected to thorough mixing by vortexing for 10 minutes and sonication for 20 minutes to ensure proper dispersion of the microplastic particles.

To evaluate the cytotoxic effects of microplastics, a series of controls and treatment groups were established:

1. Untreated cells (negative control)
2. Microplastics + Tween 20 (to assess the potential effects of the dispersant)
3. Tween 20 + cells (vehicle control)
4. Dimethyl sulfoxide (DMSO) + cells (positive control for cytotoxicity)
5. Media alone (to account for background absorbance)

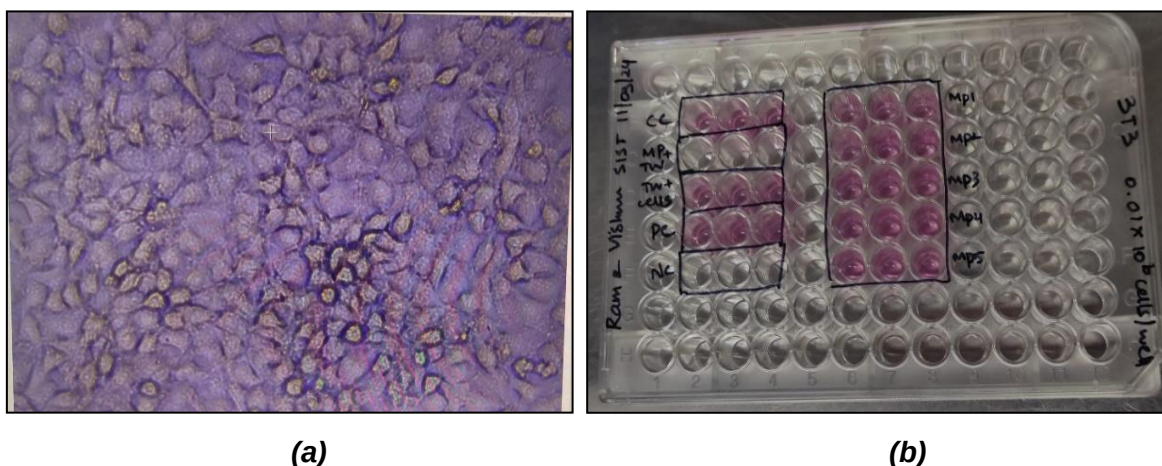


Fig 4.7: (a) Microscopic image of 3T3 cells (b) Plating of 3T3 cells in 96 well plate

The microplastic suspension was added to the designated wells at varying concentrations, ranging from 120 μ l to 200 μ l, to assess potential dose-dependent effects.

After incubating the cells with the microplastic suspensions for 48 hours, the MTT reagent was added to each well. The metabolically active cells reduced the MTT to insoluble formazan crystals, which were then solubilized by adding a solubilization buffer. This step facilitated the quantification of the formazan product, which is directly proportional to the number of viable cells.

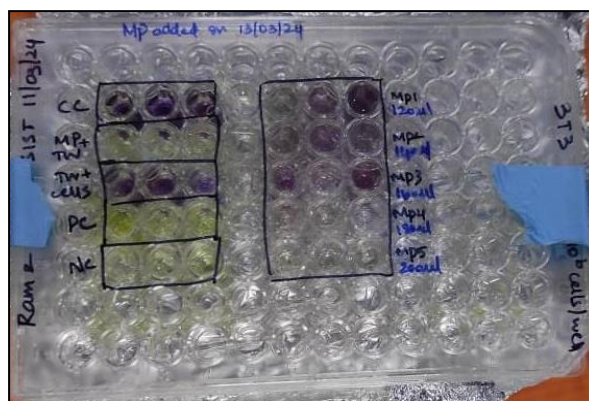


Fig 4.8: Addition of solubilization buffer

The IC50 value was calculated using the formula,

$$\% \text{ cell viability} = \frac{(\text{Abs control} - \text{Abs sample})}{\text{Abs control}} \times 100$$

$$\% \text{ cell inhibition} = 100 - \left(\frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \right) \times 100$$

4.7 STATISTICAL ANALYSIS:

For the statistical analysis of the MTT assay data, a one-way analysis of variance (ANOVA) was performed using Google Excel. The ANOVA: single factor analysis was employed to determine if there were statistically significant differences between the means of the groups being compared.

The data were organized in a spreadsheet, with rows representing the different treatment groups and columns representing the replicate values within each group. The ANOVA: single factor analysis was then conducted using the built-in function in Google Excel, which calculates the F-value and the corresponding p-value.

CHAPTER-5

5. RESULTS AND DISCUSSION:

5.1 WATER SAMPLES - OPTICAL MICROSCOPIC IMAGES:

5.1.1 NEGATIVE CONTROL:

To establish a baseline and validate the analytical method, Whatman qualitative filter paper (Grade 1, CAT No. 1440–125) was examined as a negative control prior to sample filtration. Light microscopy analysis at 4X and 10X magnification revealed no detectable impurities or microplastic particles present on the filter paper surface. The lack of contamination confirmed the suitability of the Whatman filters for use in this study, ensuring that any microplastics observed in the samples could be attributed to the sample itself rather than artifacts from the filtration medium. This crucial step allowed for accurate interpretation of the subsequent sample analysis results and reinforced the reliability of the employed methodology.

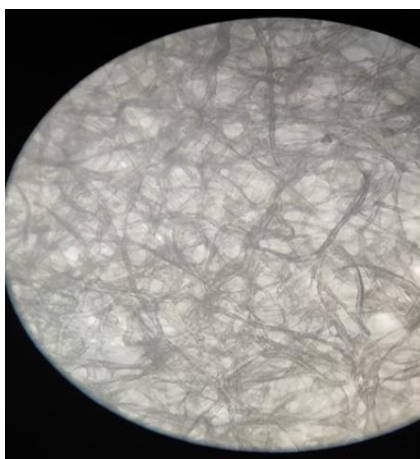


Fig 5.1: Microscopic image of filter paper as negative control

5.1.2 TAP WATER:

Upon filtering a 500-ml sample of tap water through a Whatman qualitative filter paper (Grade 1), subsequent analysis via light microscopy revealed the presence of microplastic particles and other impurities retained on the filter surface. A diverse array of microplastic morphologies was detected, including fragments, fibres, and thread-like particles exhibiting light blue, blue, and red colouration.

The microplastic fragments ranged in size from approximately 100 μm to 500 μm , with irregular shapes and jagged edges. The fibrous microplastics displayed an elongated cylindrical form, with lengths varying between 200 μm and 1000 μm . Additionally, thread-like microplastic particles were observed, appearing as thin filamentous strands interwoven amongst the other particle types.

In addition to microplastics, a significant number of particulate impurities were visibly accumulated on the filter paper. These impurities likely originated from suspended solids and other extraneous matter present in the tap water sample. The impurities exhibited varied shapes, sizes, and colourations, further complicating the visual identification and quantification of microplastic particles within the sample matrix.

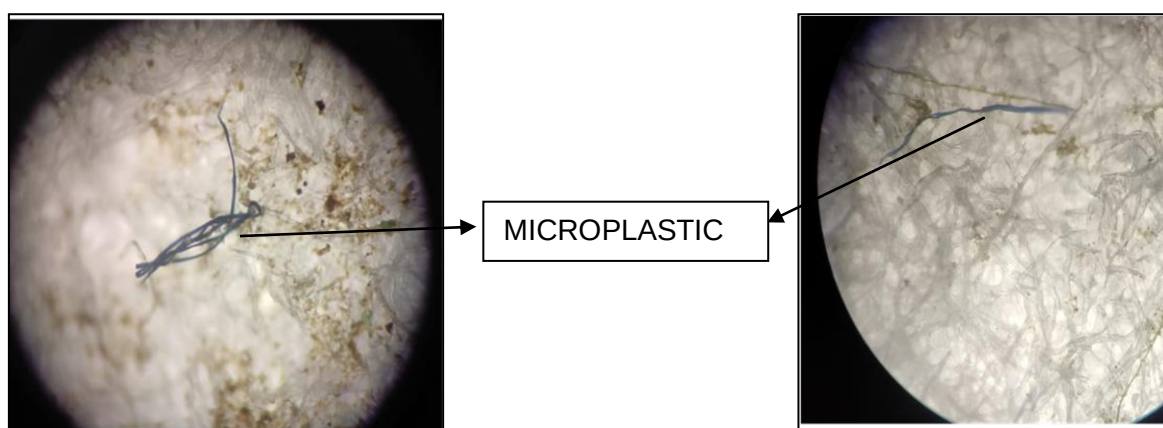


Fig 5.2: Microscopic identification of microplastics in Tap water

The microplastics and impurities were randomly distributed across the filter paper, with no apparent pattern or clustering evident. These findings highlight the pervasive nature of microplastic contamination in treated tap water supplies and underscore the importance of advanced analytical techniques for comprehensive microplastic characterization in complex environmental samples.

5.1.3 DISTILLED WATER:

Analysis via light microscopy of a 500-ml distilled water sample filtered through a Whatman qualitative filter paper (Grade 1) revealed the presence of microplastic fibres. The microplastic contaminants exhibited two distinct colourations, green and blue, with fibrous morphologies.

The green microplastic fibres were elongated and cylindrical in shape, with lengths ranging from approximately 300 μm to 1200 μm . These fibres displayed a relatively uniform diameter along their lengths, with smooth surface textures and well-defined edges. The blue microplastic fibres shared similar fibrous characteristics, though they were generally shorter in length, ranging from 200 μm to 800 μm . Both the green and blue microfibre populations exhibited considerable variation in their spatial distribution across the filter paper. Certain regions showed clusters of entangled fibres, while other areas were sparse or devoid of microplastic contamination. The microfibrils were oriented in a haphazard, randomized manner, indicating no preferential alignment.



Fig 5.3: Microscopic identification of microplastics in Distilled water

Notably, no other particulate matter or impurities were visually detected on the filter paper, suggesting a high purity of the distilled water sample. This isolated observation of coloured microplastic fibres raises concerns regarding the ubiquity of microplastic pollution, as these contaminants were present even in a highly purified water source intended for analytical applications.

5.1.4 MILLI Q WATER:

Microscopic examination of a 500-ml Milli-Q water sample filtered through a Whatman qualitative filter paper (Grade 1) revealed the presence of microplastic contaminants in the form of thin, elongated fibres and thread-like particles. A diverse array of colours, including red, blue, brown, red, and green, were represented among the microplastic particles observed.

The microplastic fibres displayed incredibly fine diametres, with most measuring less than 20 μm across. However, they exhibited considerable variation in length, ranging from approximately 200 μm to over 2000 μm for the longest fibres documented. These fibres appeared smooth, cylindrical, and unbranched along their lengths. In addition to microfibrils, microplastic particles resembling threads were also detected. These thread-like microplastics were exceptionally long and fine, with lengths frequently exceeding 1000 μm . They displayed a semi-rigid, curved morphology, often forming loose loops or tangled conformations on the filter paper.

The microplastic fibre and thread populations showed no discernible patterns in their spatial distributions across the filtered area. Particles of all colours were randomly dispersed, with some regions displaying high densities of accumulation while others remained relatively free of microplastic contamination. No other particulate matter or impurities were visibly present on the filter paper, indicating an extremely high purity of the Milli-Q water prior to filtration. This suggests that microplastic contaminants originated from an external source during water production, handling, or analytical procedures.



Fig 5.4: Microscopic identification of microplastics in Milli Q water

The microplastic fibre and thread characterizations, including dimensions, morphologies, colours, and spatial distributions, were thoroughly documented through microscopic imaging and analysis. These findings raise significant concerns regarding the extent of microplastic pollution, as even ultra-pure water intended for the most sensitive applications exhibited considerable levels of microplastic contamination.

5.1.5 RO WATER:

Light microscopic analysis of a 500-ml sample of RO (reverse osmosis) water filtered through a Whatman qualitative filter paper (Grade 1) strikingly revealed the presence of microplastic particles large enough to be visually detected without magnification. Upon carefully inspecting the filter paper, numerous vividly coloured microplastic fibres and spherical particles could be discerned by the naked eye as well.

The microplastic contaminants consisted predominantly of fibrous particles exhibiting red, blue, and green colouration, along with spherical particles in matching hues. The fibrous microplastics ranged enormously in size, with lengths spanning from an estimated 500 μm up to a remarkable 5,000 μm for the longest fibres identified. Fibre diameters also varied substantially, with some fibres exceeding 100 μm in thickness. Spherical microplastic particles were also abundantly accumulated on the filter paper, ranging in diameter from approximately 200 μm to nearly 2,000 μm for the largest spheres observed. These vividly coloured spheres displayed smooth, curved surfaces without apparent defects or irregularities.

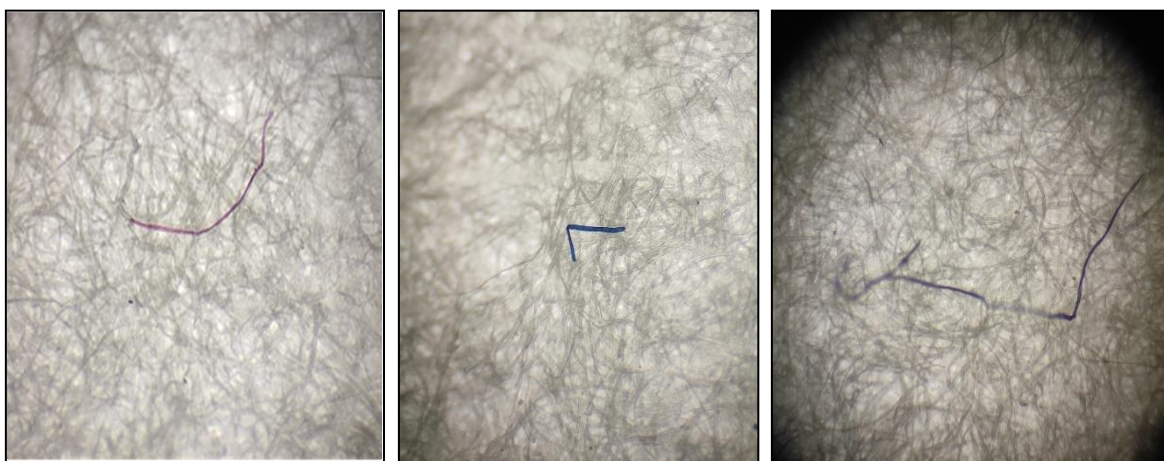


Fig 5.5: Microscopic identification of microplastics in RO water

The spatial distribution of these large microplastic particles appeared randomized, with no evident patterning or clustering across the filtered area. Certain regions were densely populated by overlapping fibres and spherical particles, while other zones remained comparatively free of visible contamination.

5.1.6 PACKAGED DRINKING WATER:

Microscopic examination of a 500-ml sample of packaged drinking water filtered through a Whatman qualitative filter paper (Grade 1) revealed the presence of microplastic contamination, predominantly in the form of small, blue fibrous particles. Interestingly, one microplastic particle exhibited a distinct, spider-like morphology. Overall, the packaged water sample displayed a relatively lower abundance of microplastics compared to other water sources analysed in this study.

The blue microplastic fibres observed ranged in length from approximately 100 μm to 500 μm , with diametres typically less than 20 μm . These fibres appeared smooth and unbranched along their lengths, with well-defined edges and a cylindrical shape. The fibres were randomly dispersed across the filtered area, with no discernible patterns in their spatial distribution. Of particular interest was the detection of a single microplastic particle with a unique, spider-like structure. This particle featured a central body region approximately 200 μm in diameter, from which multiple fibre-like projections radiated outward, resembling legs. The maximum dimension of this anomalous particle, including the leg-like projections, measured nearly 1000 μm .

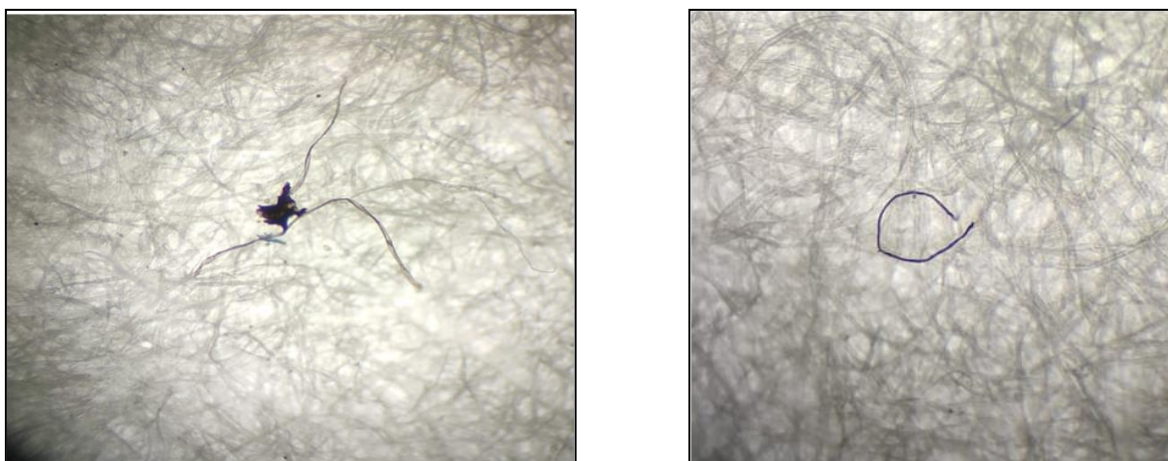


Fig 5.6: Microscopic identification of microplastics in Packaged Drinking water

Apart from the microplastic fibres and the peculiar spider-shaped particle, no other particulate contaminants or impurities were visibly present on the filter paper. Quantitative analysis revealed a substantially lower frequency of microplastic particles accumulated on the filter paper from the packaged water sample in

comparison to samples from other sources such as tap water, distilled water, and reverse osmosis water analysed in this study.

5.2 CHARACTERIZATION RESULTS:

5.2.1 RAMAN SPECTROSCOPY ANALYSIS:

5.2.1.1 REFERENCE FOR POLYETHYLENE:

To definitively identify and characterize polyethylene (PE) microplastic particles in samples using Raman spectroscopy, the reference Raman spectrum of PE serves as an indispensable tool.

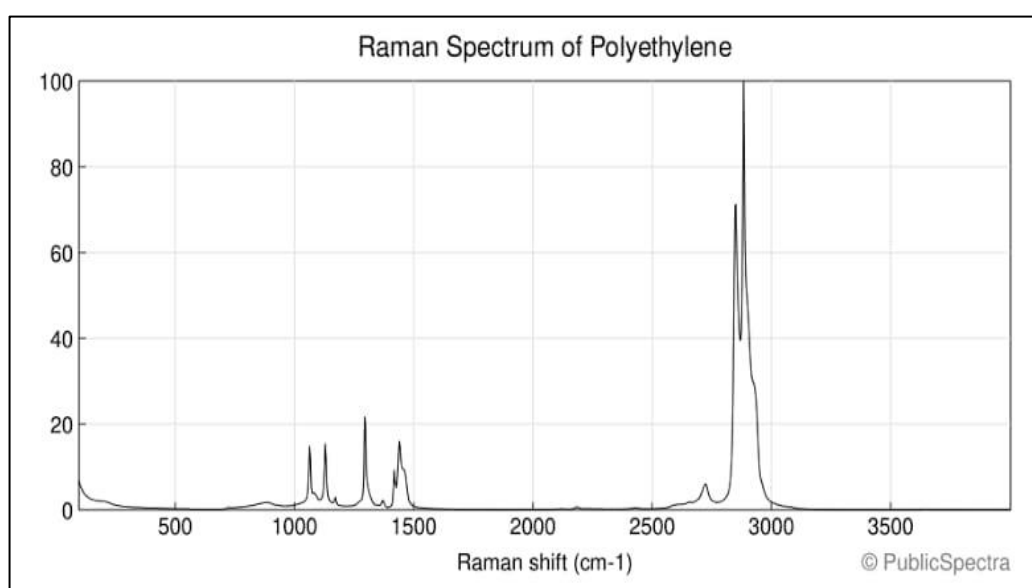


Fig 5.7: Reference Raman spectrum for Polyethylene (Lee et al., 2001)

This reference spectrum allows for the recognition of the distinct spectral fingerprints associated with the PE polymer, enabling accurate discrimination from other polymer types and sample matrix interferences. The key diagnostic peaks for PE in the Raman spectrum are located at 1125 cm⁻¹, 1290 cm⁻¹, 1410-1440 cm⁻¹, 2840 cm⁻¹, and 2870 cm⁻¹. The peaks at 1125 cm⁻¹ and 1290 cm⁻¹ arise from C-C bond vibrations within the PE backbone, while the peaks in the 1410-1440 cm⁻¹ region correspond to CH₂ bending modes. Furthermore, the peaks at 2840 cm⁻¹ and 2870 cm⁻¹ are attributed to the symmetric and asymmetric C-H stretching vibrations, respectively, of the methylene (CH₂) groups present in PE. By systematically comparing the sample Raman spectra with the PE reference and identifying the presence and relative intensities of these characteristic bands, the occurrence and

semi-quantitative abundance of PE microplastics can be reliably established within complex environmental and product samples.

5.2.1.2 REFERENCE FOR POLYETHYLENE TEREPHTHALATE:

This reference spectrum enables the recognition of the distinct spectral fingerprints associated with the PET polymer, allowing for accurate discrimination from other polymer types and sample matrix interferences. The major diagnostic peaks for PET in the Raman spectrum are located at 855, 881, 996, 1115, 1175, 1287, 1367, 1409, 1452, 1609, 1647, and 1725 cm^{-1} . Several of these peaks, such as those at 1115 cm^{-1} and 1175 cm^{-1} , arise from C-O-C stretching vibrations within the PET backbone. The peaks at 1609 cm^{-1} and 1647 cm^{-1} correspond to aromatic ring stretching modes, while the peak at 1725 cm^{-1} is attributed to C=O stretching of the ester groups.

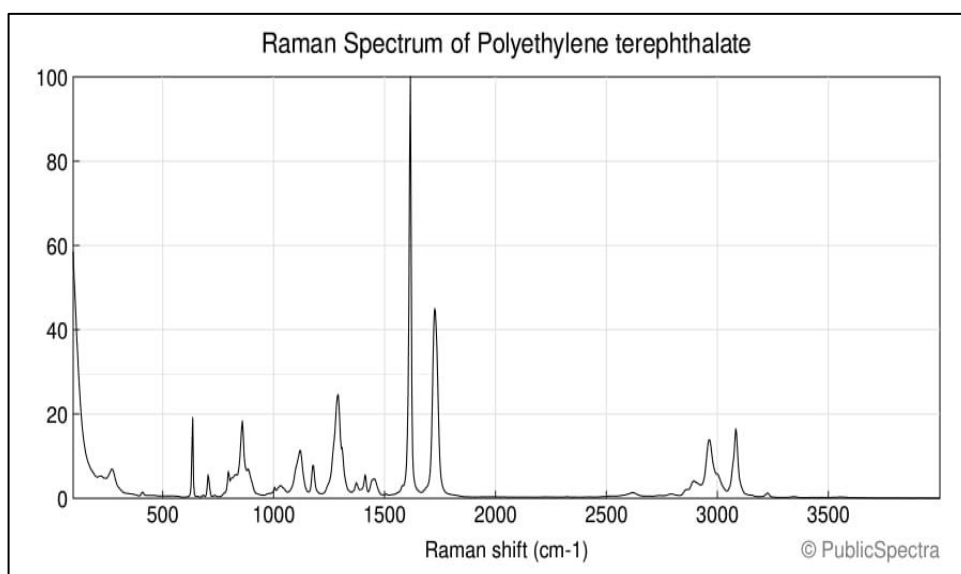


Fig 5.8: Reference Raman for Polyethylene Terephthalate (Puchowicz & Cieslak, 2022)

5.2.1.3 REFERENCE FOR POLYVINYL CHLORIDE:

This reference spectrum enables the recognition of the distinct spectral fingerprints associated with the PVC polymer, allowing for accurate discrimination from other polymer types and sample matrix components. The key diagnostic peaks for PVC in the Raman spectrum are located at 640-694 cm^{-1} , 1435 cm^{-1} , and 2914 cm^{-1} . The peak region between 640-694 cm^{-1} arises from C-Cl stretching vibrations, which are characteristic of the chlorinated backbone in PVC. The peak at 1435 cm^{-1}

¹ corresponds to C-H symmetrical stretching vibrations within the methylene (-CH₂) groups along the PVC chain. Additionally, the peak at 2914 cm⁻¹ is attributed to C-H stretching modes of these methylene groups.

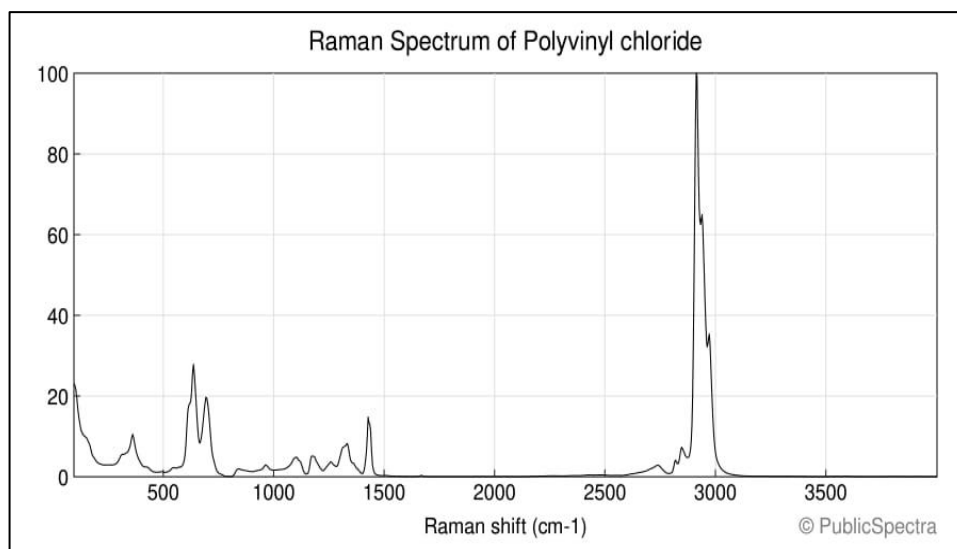


Fig 5.9: Reference Raman for Polyvinyl chloride (Prokhorov et al., 2016)

5.2.1.4 TAP WATER:

The analysis of the obtained Raman spectrum provides valuable insights into the chemical composition and identification of the microplastic sample under investigation. The spectrum revealed several prominent peaks that can be correlated with the characteristic vibrational modes of polyethylene, a widely used plastic polymer.

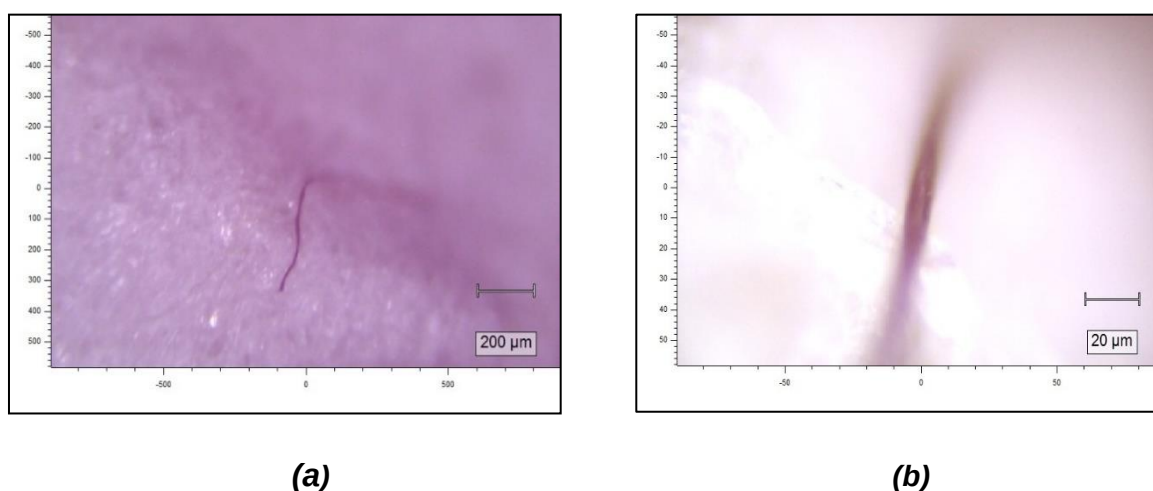


Fig 5.10: Optical microscopic image taken by Raman for Tap water in 5X and 50X

Notably, the peaks observed at 1137, 1159, and 1187 cm^{-1} correspond to the C-C stretching region, indicating the presence of crystalline phases of PE. This region is particularly distinctive for PE and serves as a reliable marker for its identification. Furthermore, the peak at 1289 cm^{-1} is consistent with the CH_2 twisting vibration typically observed in PE. Additionally, the peaks at 1348 and 1414 cm^{-1} align with the characteristic CH_2 bending vibrations associated with the PE polymer structure.

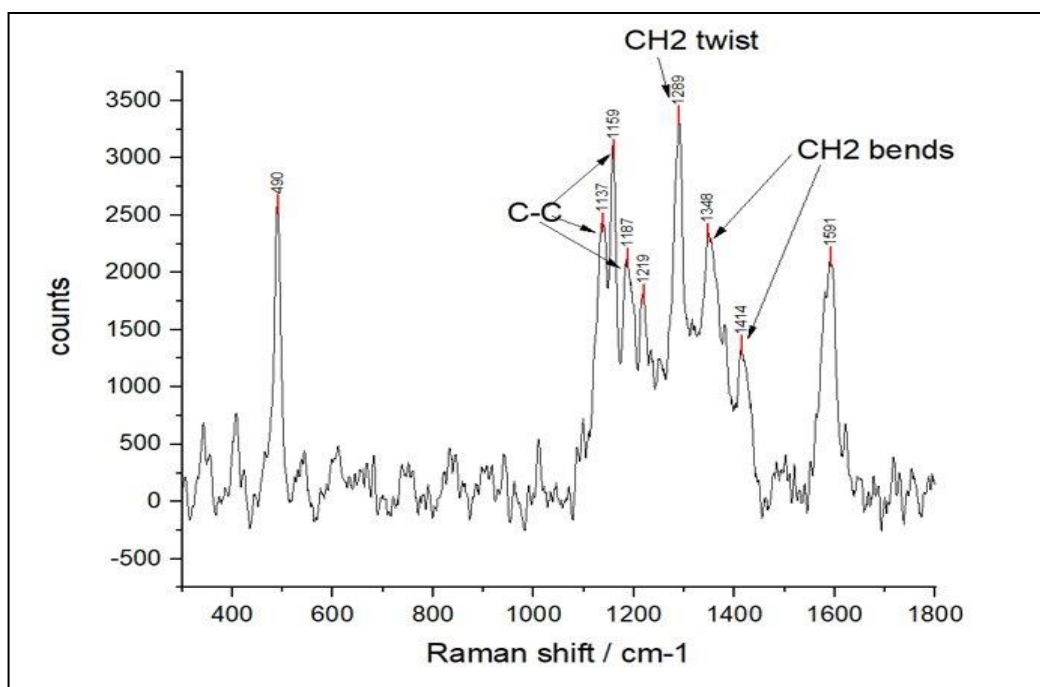


Fig 5.11: Raman spectra of identified polymer in Tap water

The presence of multiple characteristic peaks related to C-C stretching, CH_2 bending, and CH_2 scissoring vibrations in the obtained Raman spectrum strongly supports the identification of the microplastic sample as polyethylene. The observed spectral features closely match the reference spectra for PE, providing a high degree of confidence in the assignment of the polymer type.

5.2.1.5 DISTILLED WATER:

Notably, the peaks observed at 1138, 1158, and 1185 cm^{-1} correspond to the C-C stretching region, which is a distinctive feature of PE. The presence of these peaks suggests the existence of crystalline phases within the PE structure of the sample.

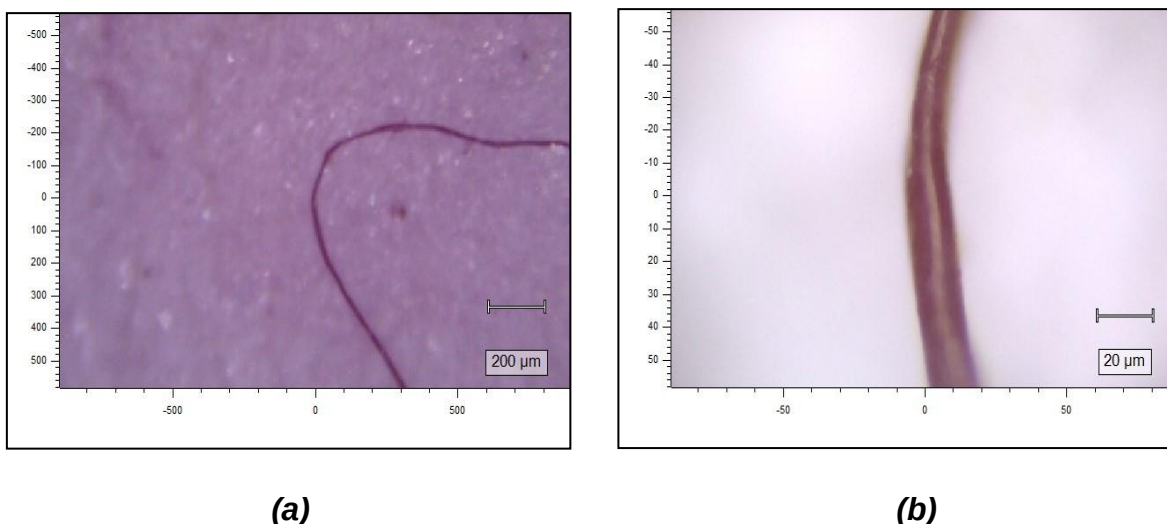


Fig 5.12: (a), (b) Optical microscopic image taken by Raman for Distilled water in 5X and 50X

Furthermore, the peak at 1289 cm^{-1} is consistent with the CH_2 twisting vibration typically observed in PE molecules. Additionally, the peaks at 1352 , 1382 , and 1419 cm^{-1} align with the characteristic CH_2 bending vibrations associated with the PE polymer structure. The observation of multiple characteristic peaks related to C-C stretching, CH_2 bending, and CH_2 scissoring vibrations in the Raman spectrum strongly supports the identification of the microplastic sample as polyethylene.

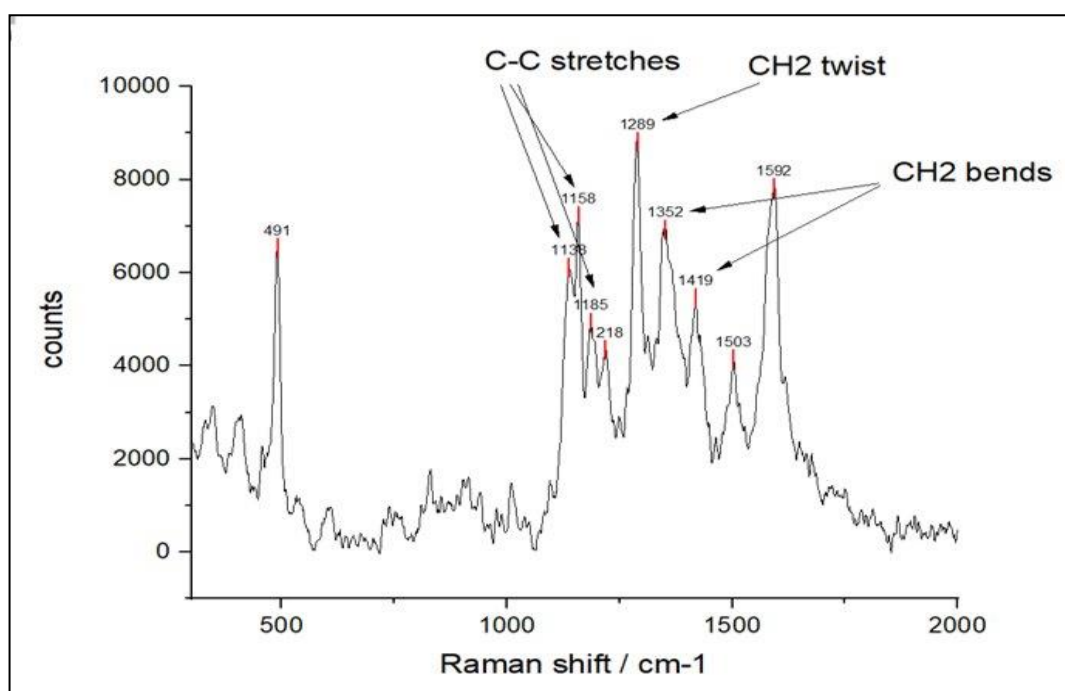


Fig 5.13: Raman spectra of identified polymer in Distilled water

These spectral features closely match the reference spectra for PE, providing a high degree of confidence in the assignment of the polymer type.

5.2.1.6 MILLI Q WATER:

Notably, the peaks observed at 600 cm^{-1} and 762 cm^{-1} are attributed to C-Cl stretching vibrations, which are distinctive features of PVC. The presence of these peaks suggests the presence of chlorine atoms in the polymer structure, a characteristic of PVC. Additionally, the peaks at 1232 cm^{-1} and 1316 cm^{-1} correspond to CH_2 bending modes, while the peaks at 1369 cm^{-1} and 1466 cm^{-1} are indicative of CH_2 scissoring vibrations, which are characteristic of the aliphatic hydrocarbon backbone of PVC.

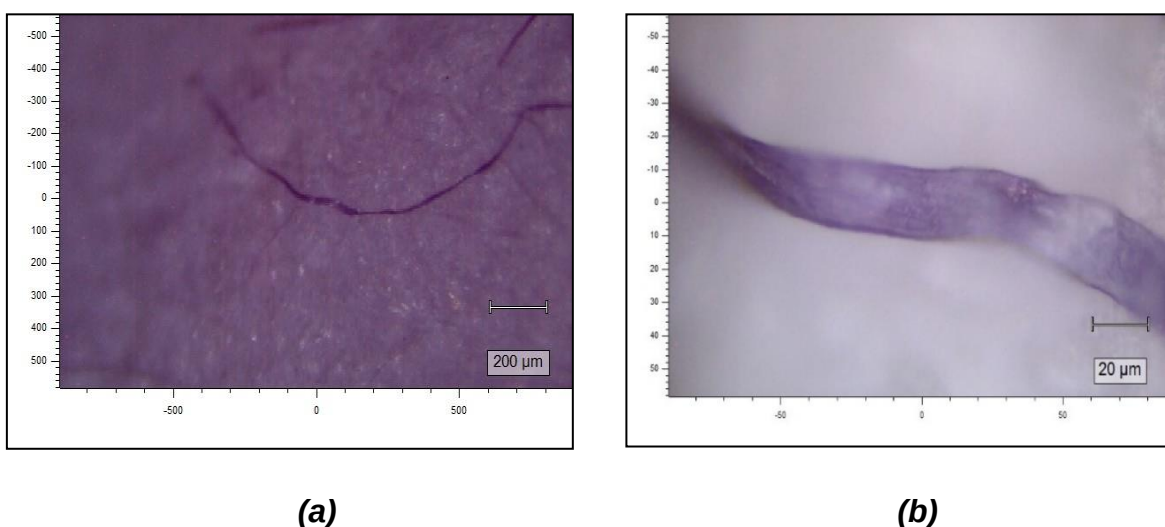


Fig 5.14: (a), (b) Optical microscopic image taken by Raman for Milli Q water in 5X and 50X

Despite the absence of peaks in the C-H stretch range in the obtained spectrum, the presence of multiple intensive peaks attributable to C-Cl vibrations around $600\text{--}700\text{ cm}^{-1}$ and CH_2 scissor/bend modes from $1200\text{--}1500\text{ cm}^{-1}$ aligns exceptionally well with the literature on PVC Raman shifts. This correlation strongly suggests that the analysed sample is PVC, as these specific vibrational modes are characteristic of such material.

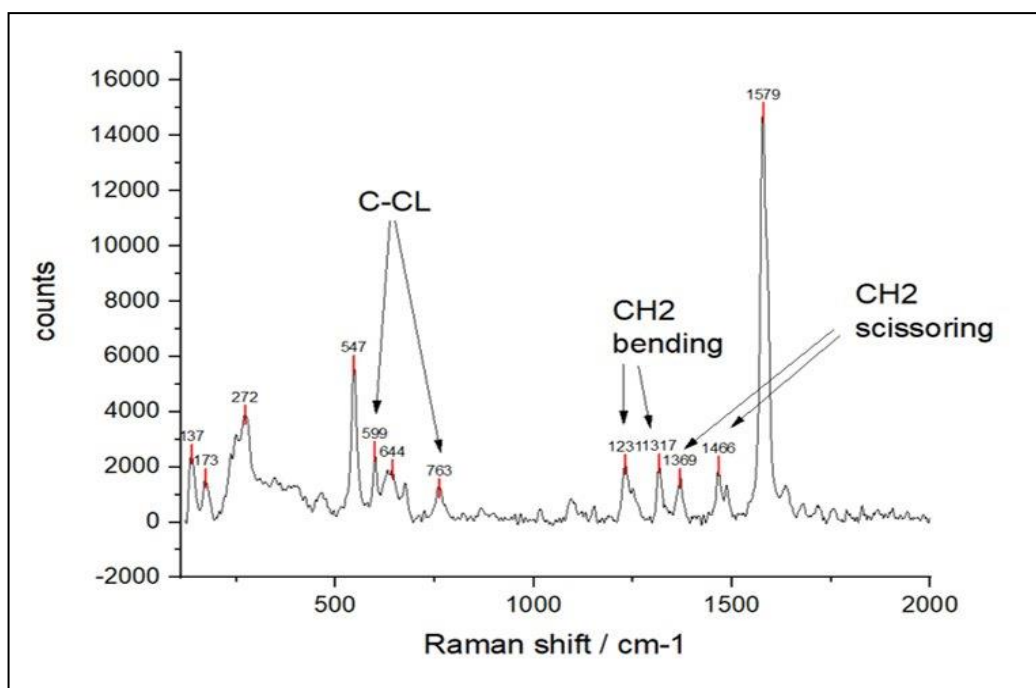


Fig 5.15: Raman spectra of identified polymer in Milli Q water

5.2.1.7 RO WATER:

Notably, the peaks observed at 1080 cm^{-1} , 1168 cm^{-1} , and 1200 cm^{-1} are attributed to C-O stretching vibrations in aliphatic chains, which are characteristic of the polyethylene component of PET. Additionally, the peak at 1395 cm^{-1} corresponds to the CH_2 scissoring bending mode, further supporting the presence of aliphatic components.

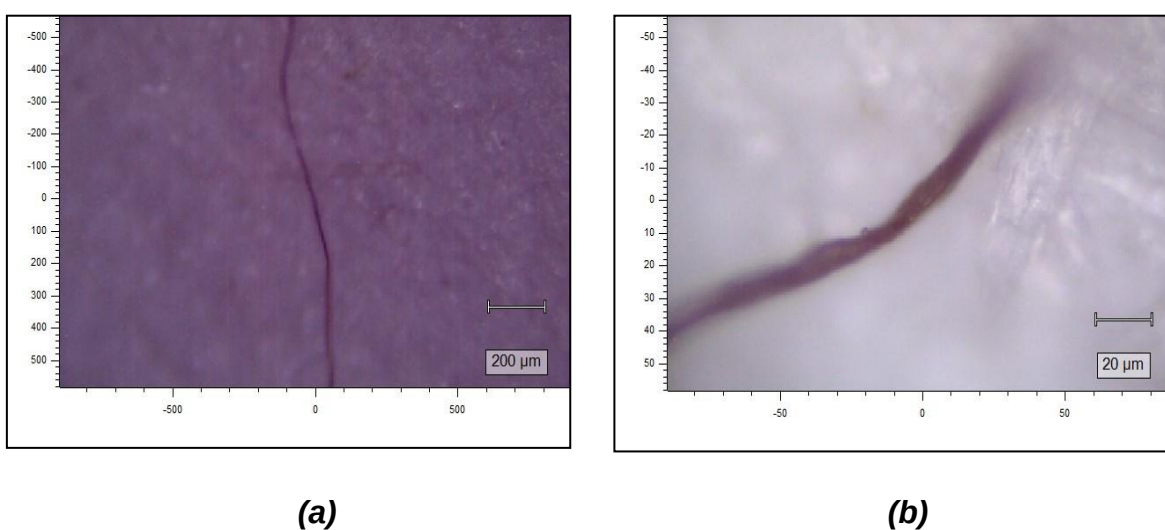


Fig 5.16: (a), (b) Optical microscopic image taken by Raman for RO water in 5X and 50X

Furthermore, the peaks at 1585 cm^{-1} and 1619 cm^{-1} are associated with the vibrational modes of aromatic rings, which can be attributed to the terephthalate component of PET. The strong peak observed at 1729 cm^{-1} is indicative of the C=O stretching vibration, which is a characteristic feature of the ester groups present in PET.

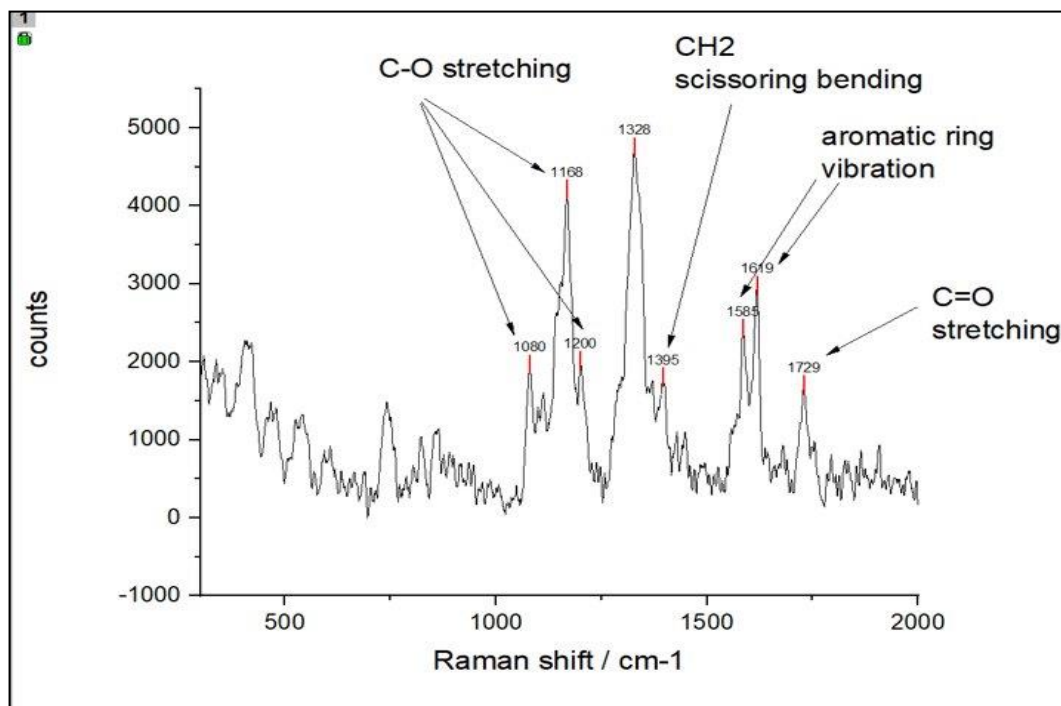


Fig 5.17: Raman spectra of identified polymer in RO water

The presence of a significant carbonyl peak at 1729 cm^{-1} , alongside the aromatic peaks (1585 cm^{-1} and 1619 cm^{-1}) and aliphatic C-O stretches, strongly indicates that the analysed sample is indeed polyethylene terephthalate. This identification is consistent with the chemical composition of PET, which includes both aromatic terephthalate and aliphatic ethylene components, matching the observed mix of vibrational modes and their respective peaks.

5.2.1.8 PACKAGED DRINKING WATER:

Notably, the peaks observed at 1080 cm^{-1} and 1170 cm^{-1} are attributed to C-O stretching vibrations in aliphatic chains, which are characteristic of the polyethylene component of PET. Additionally, the peak at 1328 cm^{-1} corresponds to the ring-breathing mode, further supporting the presence of aromatic components.

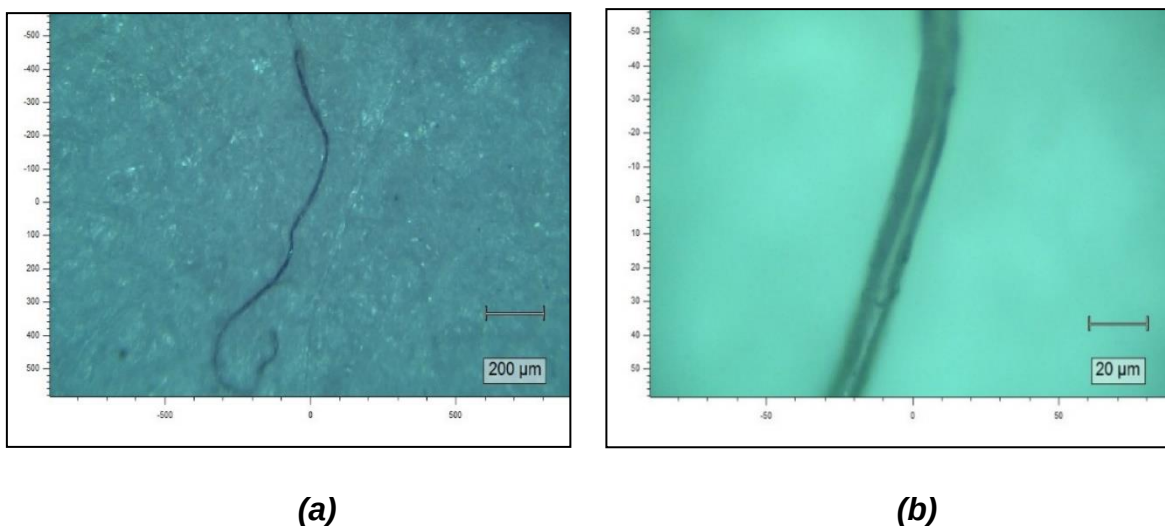


Fig 5.18: (a), (b) Optical microscopic image taken by Raman for Packaged drinking water in 5X and 50X

Furthermore, the peaks at 1586 cm^{-1} and 1618 cm^{-1} are associated with the vibrational modes of aromatic rings, which can be attributed to the terephthalate component of PET. The strong peak observed at 1731 cm^{-1} is indicative of the C=O stretching vibration, which is a characteristic feature of the ester groups present in PET.

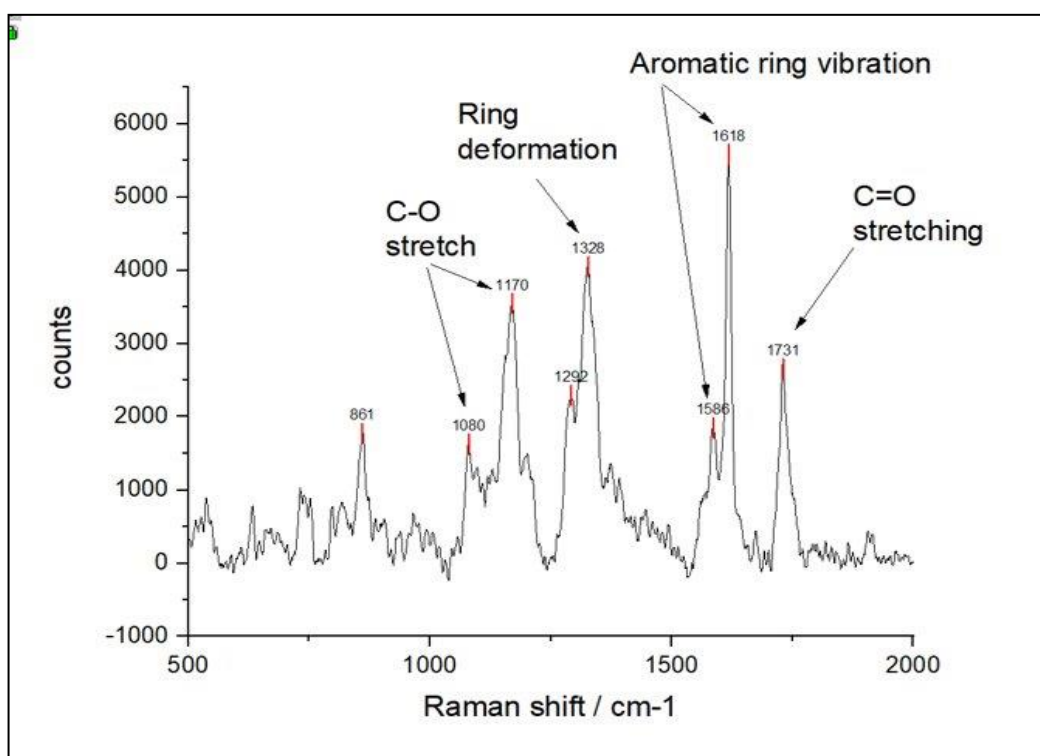


Fig 5.19: Raman spectra of identified polymer in Packaged drinking water

The presence of a significant carbonyl peak at 1731 cm^{-1} , alongside the aromatic peaks (1586 cm^{-1} and 1618 cm^{-1}) and aliphatic C-O stretches, strongly indicates that the analysed sample is indeed polyethylene terephthalate. This identification is consistent with the chemical composition of PET, which includes both aromatic terephthalate and aliphatic ethylene components, matching the observed mix of vibrational modes and their respective peaks.

5.3 MILK SAMPLES - OPTICAL MICROSCOPIC IMAGES:

5.3.1 M1 SAMPLE:

Microscopic examination of the filtered milk sample M1 revealed the presence of microplastic contaminants exhibiting diverse colours, morphologies, and sizes amidst other particulate components inherent to the milk matrix. Vividly coloured red, blue, and green microplastics were detected, along with a distinct brown microplastic particle displaying a spindle-like shape.



Fig 5.20: Microscopic identification of microplastics in M1 sample

The red, blue, and green microplastics observed consisted primarily of fibrous particles and fragments. The fibrous microplastics ranged in length from approximately $200\text{ }\mu\text{m}$ to $1000\text{ }\mu\text{m}$, with diameters typically between 10 and $30\text{ }\mu\text{m}$. The fragments varied significantly in size and shape, with dimensions spanning $50\text{ }\mu\text{m}$ to $500\text{ }\mu\text{m}$ across their maximum axes. Of particular interest was the observation of a brown, spindle-shaped microplastic particle. This particle featured an elongated, fusiform morphology with tapered ends and a slightly curved midsection. Its maximum length was measured at approximately $800\text{ }\mu\text{m}$, with a maximum width of

150 μm at the broadest point. The spindle-shaped particle displayed a smooth surface texture without apparent irregularities.

The microplastic particles were randomly distributed across the filtered area, often in proximity to or entangled with other particulate matter characteristic of the milk sample, such as protein agglomerates, fat globules, and cellular debris. No discernible patterns or clustering of the microplastics were evident. Comprehensive microscopic imaging and analysis thoroughly documented the physical characteristics of the microplastic contaminants, including dimensions, morphologies, colours, and spatial distributions. The co-occurrence and interactions between microplastics and other milk particulates were also meticulously recorded to elucidate the complexity of microplastic contamination within food matrices.

These findings highlight the diverse range of microplastic particles that can be present in dairy products, encompassing various shapes, sizes, and colourations. The detection of the distinct brown spindle-shaped microplastic emphasizes the importance of rigorous analytical techniques to fully characterize the breadth of microplastic contamination in food and environmental samples.

5.3.2 M2 SAMPLE:

Light microscopic analysis of the filtered milk sample M2 revealed the presence of microplastic contaminants among other particulate matter originating from the milk matrix itself. The microplastics observed exhibited diverse morphologies, sizes, and colourations, including blue fibres with thread-like ends, orange long fibres, and light blue microplastic particles.

The blue microplastic fibres displayed a distinct morphology, with cylindrical fibre bodies transitioning into finer, thread-like extremities at each end. The fibre bodies ranged in length from approximately 300 μm to 800 μm , with diameters around 20–30 μm . However, the thread-like ends were significantly finer, often less than 10 μm in diameter, and extended an additional 100–300 μm from the main fibre body. Orange microplastic fibres were also detected, appearing as elongated, smoothly cylindrical particles without branching or irregularities. These orange fibres were remarkably long, with lengths frequently exceeding 1000 μm and some particles measuring up to 2000 μm . Their diameters were relatively consistent, typically ranging between 15 and 25 μm .



Fig 5.21: Microscopic identification of microplastics in M2 sample

In addition to the fibrous microplastics, light blue microplastic particles of various morphologies were observed, including fragments, films, and amorphous shapes. These light blue microplastics varied significantly in size, with dimensions spanning from approximately 50 μm to 500 μm across their maximum axes. The microplastic particles were randomly distributed across the filtered area, with no discernible patterns or clustering evident. In several regions, the microplastics appeared entangled or interspersed with other particulate matter characteristic of the milk sample, such as protein agglomerates, fat globules, and cellular debris.

5.3.3 M3 SAMPLE:

Light microscopic analysis of the filtered M3 milk sample revealed a diverse array of microplastic contaminants exhibiting striking morphologies, colours, and sizes intermixed with other particulate components inherent to the milk matrix. Notable microplastic particles observed included blue flower-like structures, a black thick fibre, red snake-like particles, and light blue and green microplastics of varying shapes.

The blue flower-like microplastic particles displayed a distinctive radial morphology, with multiple thin fibrous projections emanating from a central nodule. The overall diameter of these floral structures ranged from approximately 200 μm to 500 μm , with the individual fibrous projections extending 100–300 μm outward. A remarkably thick black microplastic fibre was also detected, with a diameter exceeding 100 μm and a length of nearly 2000 μm . This fibre appeared smooth and unbranched along its length, exhibiting a consistent cylindrical morphology.

Red microplastic particles with a striking snake-like appearance were present, featuring elongated, curved, and intertwined morphologies. These sinuous particles ranged in length from 500 μm to 1500 μm , with widths varying between 50 and 150 μm along their contorted forms. In addition to the distinctive microplastics described above, light blue and green microplastic particles of various morphologies, including fibres, fragments, and films, were abundantly observed. These particles spanned a broad size range, with dimensions ranging from approximately 50 μm to 1000 μm .



Fig 5.22: Microscopic identification of microplastics in M3 sample

The microplastic particles were randomly dispersed across the filtered area, often in proximity to or entangled with other milk particulates such as protein agglomerates, fat globules, and cellular debris. No discernible patterns or clustering were evident.

5.3.4 M4 SAMPLE:

Microscopic examination of the filtered M4 milk sample revealed the presence of microplastic fibre contaminants, predominantly comprising blue and red fibres. Notably, the red microplastic fibres observed were exceptionally long in length. Overall, the M4 sample exhibited a relatively low abundance of microplastic particles compared to other milk samples analysed in this study. The microplastic fibres were detected amidst other particulate matter characteristic of the milk matrix. The blue microplastic fibres ranged in length from approximately 200 μm to 800 μm , with diameters typically between 10-20 μm . These fibres appeared smooth and unbranched along their lengths, exhibiting a consistent cylindrical morphology.

In contrast, the red microplastic fibres were remarkably long, with lengths frequently exceeding 1000 μm and some fibres measuring up to 3000 μm . Despite their considerable lengths, the red fibres maintained relatively uniform diameters, typically between 15-25 μm . These fibres displayed well-defined edges and a smooth surface texture. Both the blue and red microplastic fibre populations were randomly distributed across the filtered area, with no discernible patterns or clustering evident. The fibres were often in close proximity or intertwined with other milk particulates such as protein agglomerates, fat globules, and cellular debris.



Fig 5.23: Microscopic identification of microplastics in M4 sample

Quantitative analysis revealed a substantially lower frequency of microplastic particles present in the M4 milk sample compared to other milk samples examined in this study. This reduced microplastic load was visually apparent during microscopic observation, with larger areas of the filter paper appearing relatively free of microplastic contamination.

5.4 CHARACTERIZATION RESULTS:

5.4.1 RAMAN SPECTROSCOPY ANALYSIS:

5.4.1.1 M1 SAMPLE:

Notably, the peaks observed at 1137, 1159, and 1183 cm^{-1} correspond to the C-C stretching region, indicating the presence of crystalline phases within the PE structure of the sample. This region is particularly distinctive for PE and serves as a reliable marker for its identification. Furthermore, the peak at 1289 cm^{-1} is consistent with the CH_2 twisting vibration typically observed in PE molecules. Additionally, the

peaks at 1349 and 1415 cm^{-1} align with the characteristic CH_2 bending vibrations associated with the PE polymer structure.

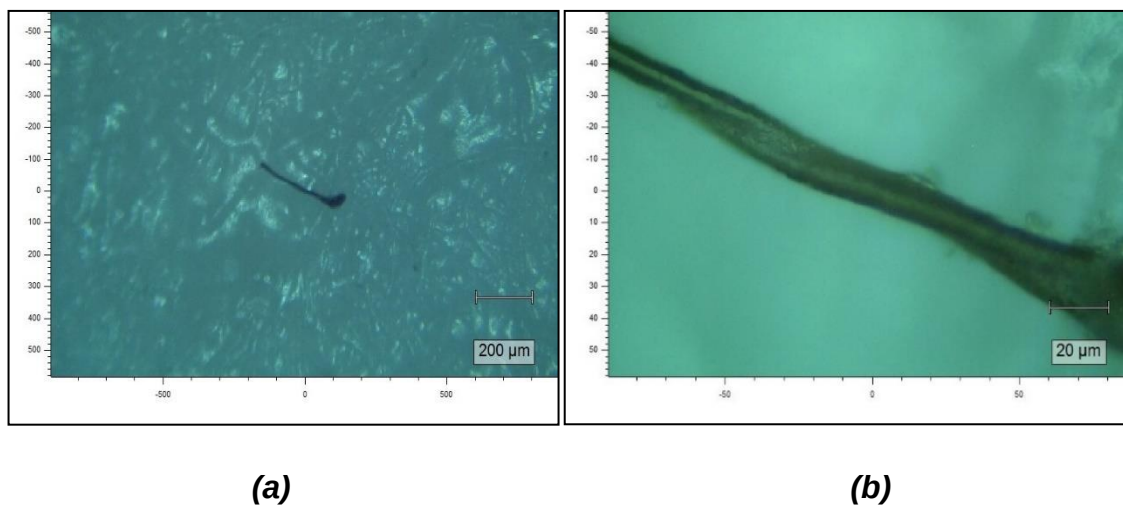


Fig 5.24: (a), (b) Optical microscopic image taken by Raman for M1 sample in 5X and 50X

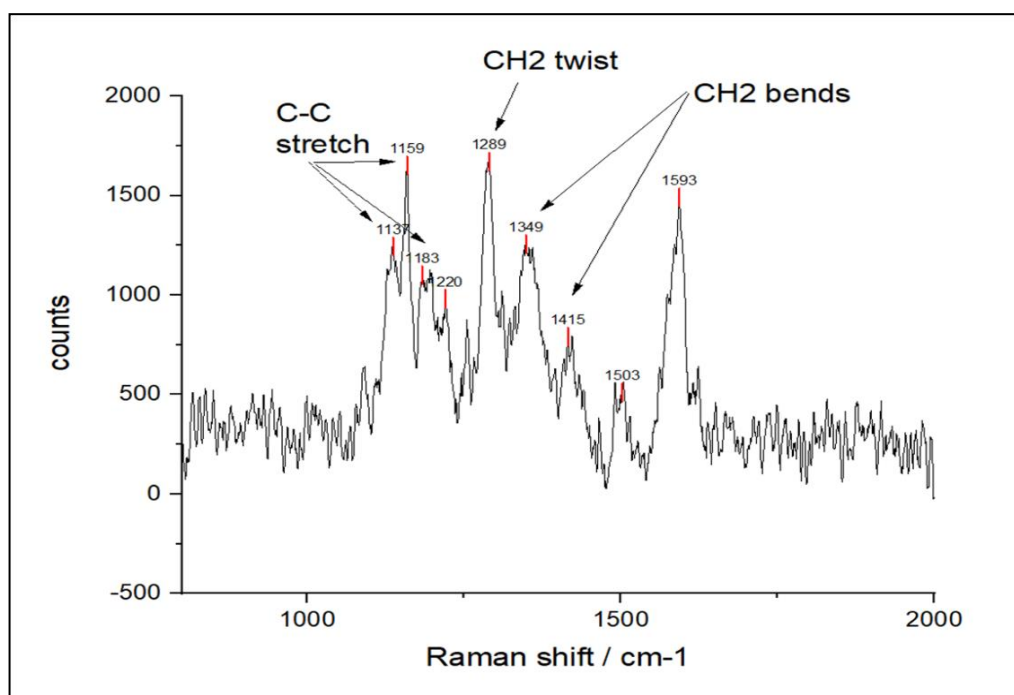


Fig 5.25: Raman spectra of identified polymer in M1 sample

The presence of multiple characteristic peaks related to C-C stretching, CH_2 bending, and CH_2 scissoring vibrations in the obtained Raman spectrum strongly supports the identification of the microplastic sample as polyethylene. These

spectral features closely match the reference spectra for PE, providing a high degree of confidence in the assignment of the polymer type.

5.4.1.2 M2 SAMPLE:

Notably, the peaks observed at 1132, 1159, and 1191 cm^{-1} correspond to the C-C stretching region, indicating the presence of crystalline phases within the PE structure of the sample. This region is particularly distinctive for PE and serves as a reliable marker for its identification.

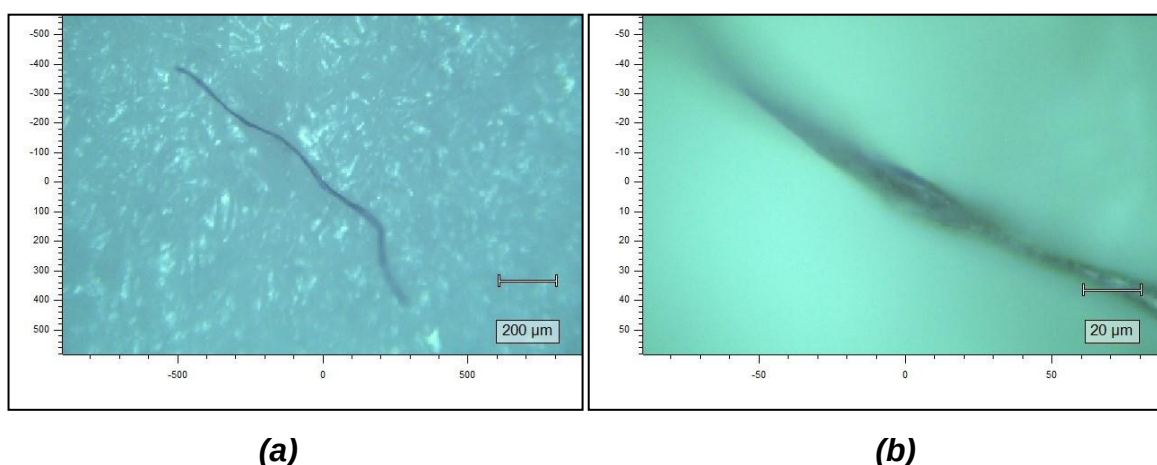


Fig 5.26: (a), (b) Optical microscopic image taken by Raman for M2 sample in 5X and 50X

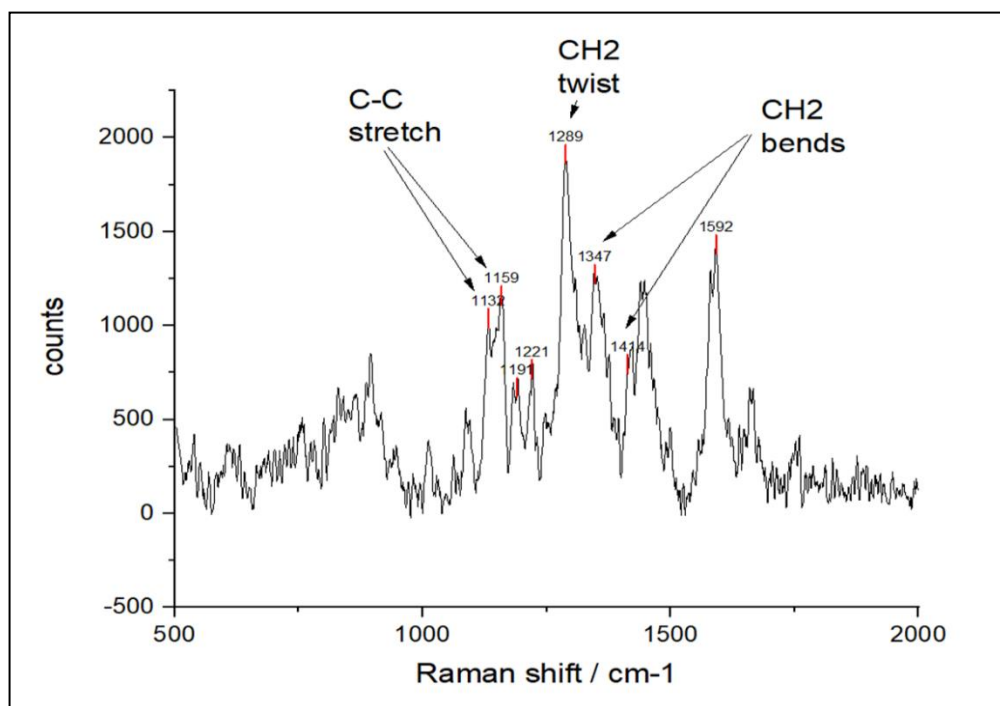


Fig 5.27: Raman spectra of identified polymer in M2 sample

Furthermore, the peak at 1289 cm^{-1} is consistent with the CH_2 twisting vibration typically observed in PE molecules. Additionally, the peaks at 1347 and 1414 cm^{-1} align with the characteristic CH_2 bending vibrations associated with the PE polymer structure. The presence of multiple characteristic peaks related to C-C stretching, CH_2 bending, and CH_2 scissoring vibrations in the obtained Raman spectrum strongly supports the identification of the microplastic sample as polyethylene. These spectral features closely match the reference spectra for PE, providing a high degree of confidence in the assignment of the polymer type.

5.4.1.3 M3 SAMPLE:

Notably, the peaks observed at 1132 , 1155 , and 1187 cm^{-1} correspond to the C-C stretching region, indicating the presence of crystalline phases within the PE structure of the sample.

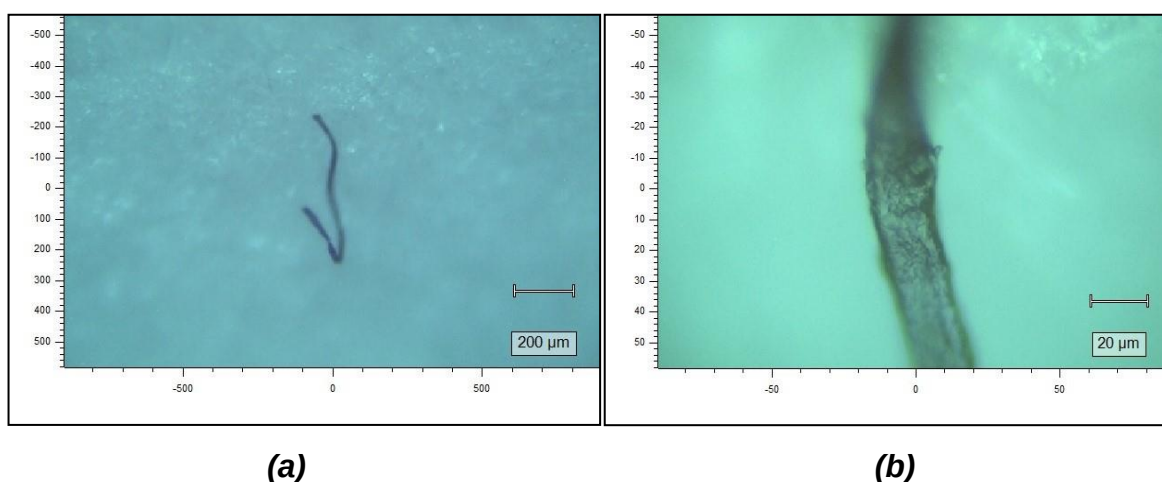


Fig 5.28: (a), (b) Optical microscopic image taken by Raman for M3 sample in 5X and 50X

This region is particularly distinctive for PE and serves as a reliable marker for its identification. Furthermore, the peak at 1287 cm^{-1} is consistent with the CH_2 twisting vibration typically observed in PE molecules. Additionally, the peaks at 1348 and 1410 cm^{-1} align with the characteristic CH_2 bending vibrations associated with the PE polymer structure. The presence of multiple characteristic peaks related to C-C stretching, CH_2 bending, and CH_2 scissoring vibrations in the obtained Raman spectrum strongly supports the identification of the microplastic sample as polyethylene. These spectral features closely match the reference spectra for PE, providing a high degree of confidence in the assignment of the polymer type.

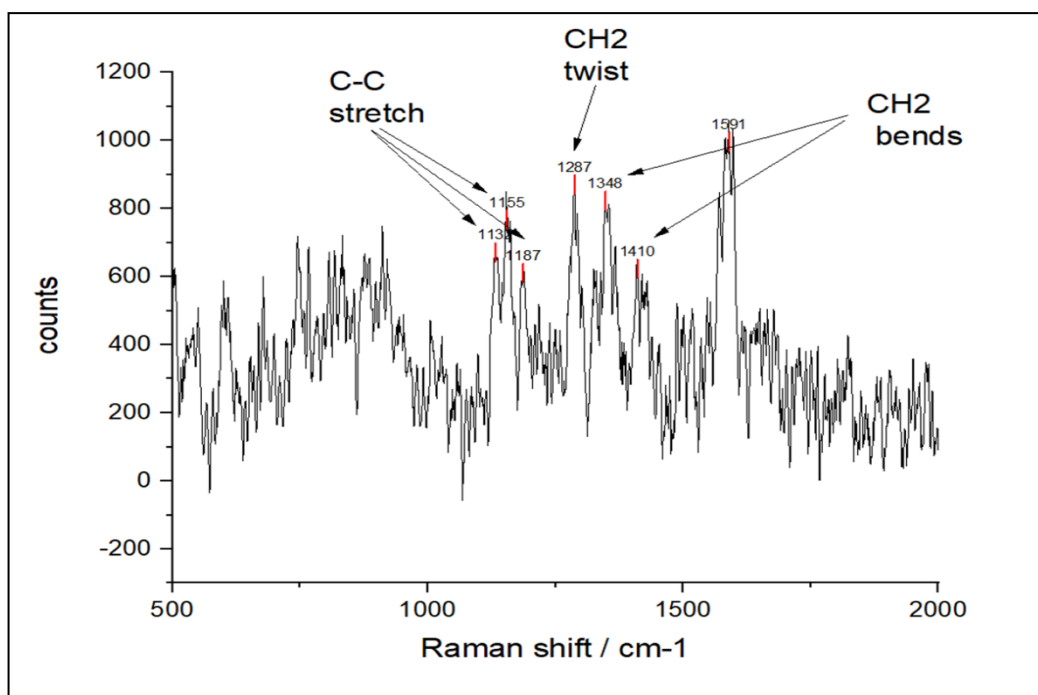


Fig 5.29: Raman spectra of identified polymer in M3 sample

5.4.1.4 M4 SAMPLE:

Notably, the peaks observed at 1142, 1157, and 1186 cm^{-1} correspond to the C-C stretching region, indicating the presence of crystalline phases within the PE structure of the sample. This region is particularly distinctive for PE and serves as a reliable marker for its identification. Furthermore, the peak at 1287 cm^{-1} is consistent with the CH_2 twisting vibration typically observed in PE molecules. Additionally, the peaks at 1348 and 1416 cm^{-1} align with the characteristic CH_2 bending vibrations associated with the PE polymer structure.

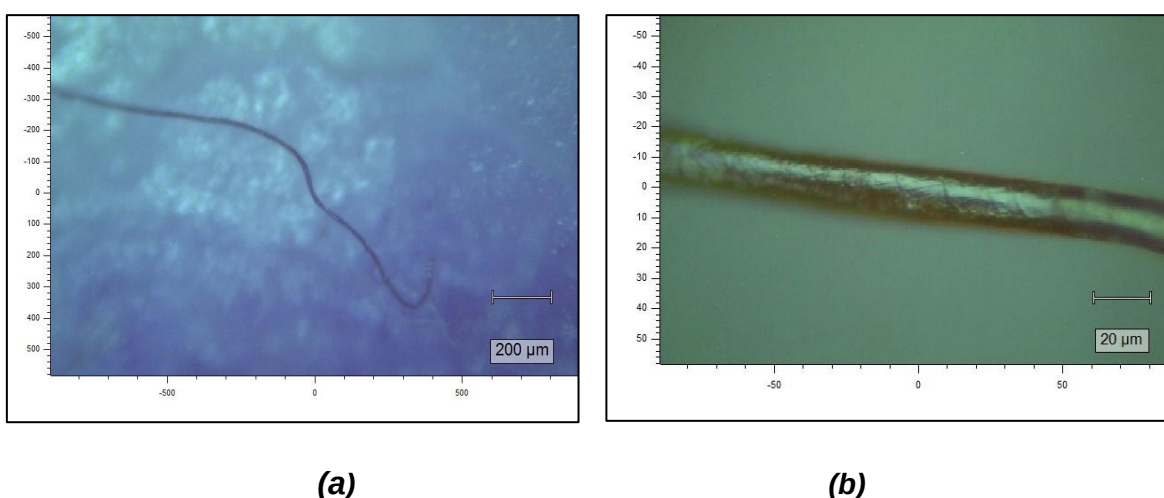


Fig 5.30: Optical microscopic image taken by Raman for M4 sample

The presence of multiple characteristic peaks related to C-C stretching, CH₂ bending, and CH₂ scissoring vibrations in the obtained Raman spectrum strongly supports the identification of the microplastic sample as polyethylene. These spectral features closely match the reference spectra for PE, providing a high degree of confidence in the assignment of the polymer type.

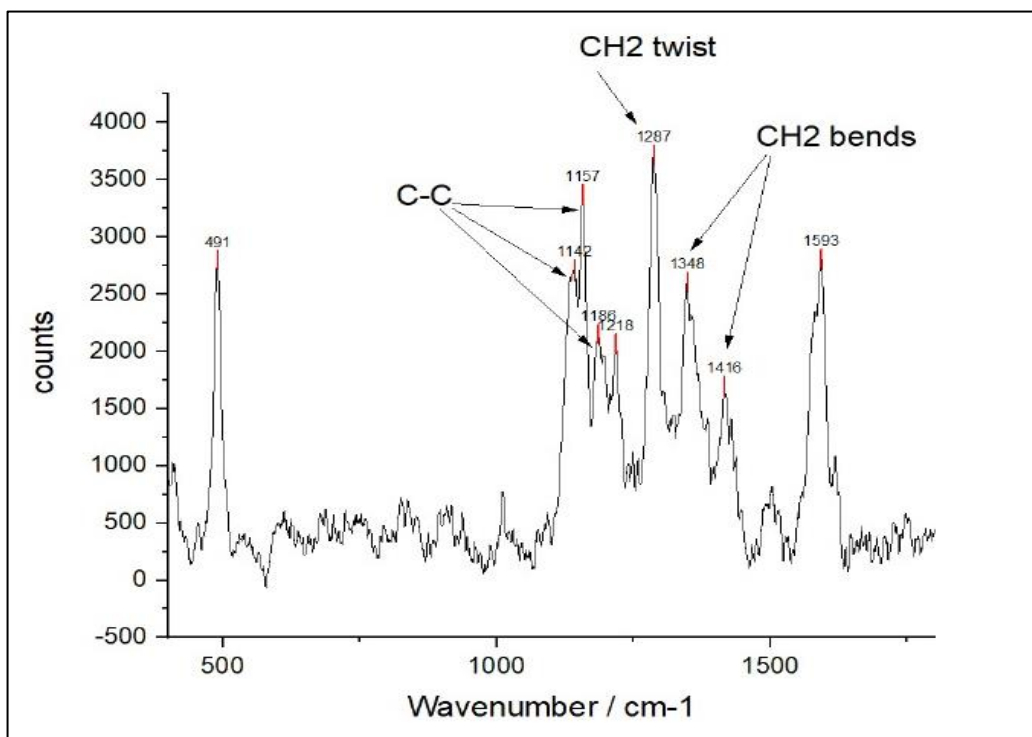


Fig 5.31: Raman spectra of identified polymer in M4 sample

5.4.2 FTIR ANALYSIS:

5.4.2.1 REFERENCE FOR MILK:

Interpreting the FTIR spectra of milk samples potentially contaminated with microplastics requires a comprehensive understanding of the characteristic spectral features arising from the milk matrix itself. A reference FTIR spectrum of pure cow's milk serves as an invaluable tool for recognizing and subtracting these milk-derived peaks, thereby isolating the spectral signatures corresponding to any extraneous microplastic particles present. The broad peak observed around 3000 cm⁻¹ in the milk spectrum is attributed to the O-H stretching vibrations of water molecules and hydroxyl groups found in milk components such as lactose and proteins. Additionally, the region between 2800-3000 cm⁻¹ exhibits peaks resulting from the

asymmetric and symmetric C-H stretching vibrations of methyl ($-\text{CH}_3$) and methylene ($-\text{CH}_2-$) groups, which are prevalent in the fatty acid components of milk lipids.

Furthermore, two distinct peaks are characteristic of the protein fractions in milk. The peak around 1650 cm^{-1} corresponds to the amide I band, arising from C=O stretching vibrations of the peptide bonds in milk proteins. Complementing this, the peak at approximately 1540 cm^{-1} is assigned to the amide II band, which originates from the coupling of N-H bending and C-N stretching modes within the protein backbone. By systematically identifying and cancelling these milk-specific spectral features from the sample spectra, the remaining peaks can be deconvoluted and assigned to microplastic contaminants, enabling their reliable identification and characterization within the complex milk matrix. This crucial step ensures accurate interpretation of the spectral data and reinforces the reliability of the microplastic analysis in milk and other food products.

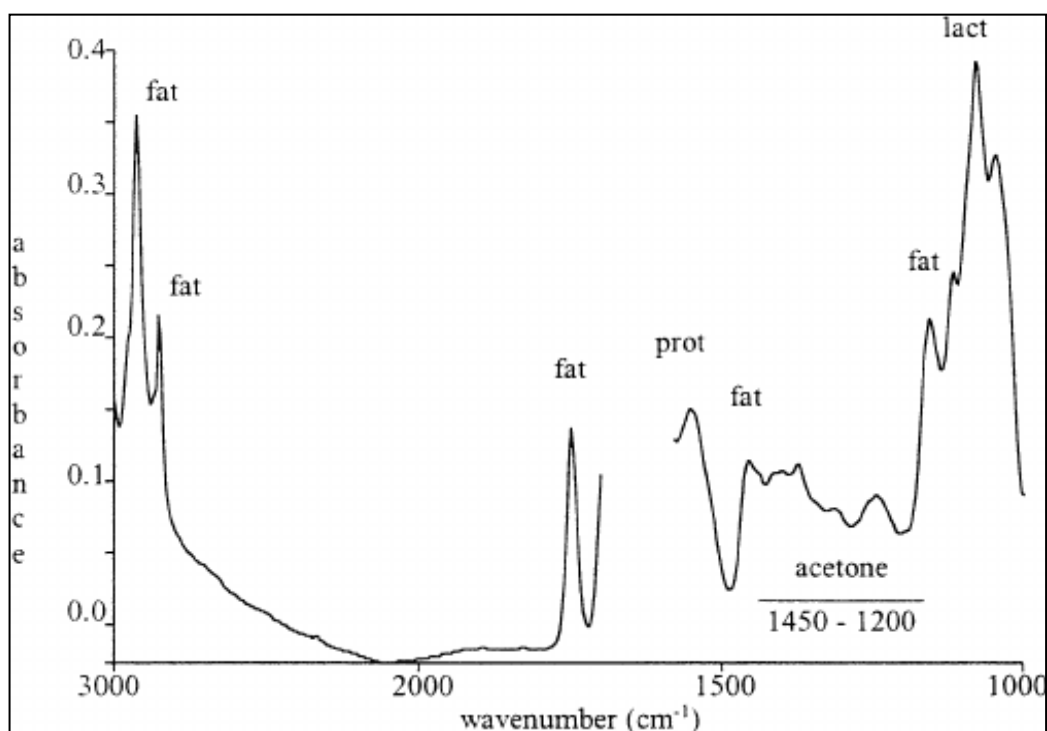


Fig 5.32: Reference FTIR spectrum of milk (Heuer et al., 2001)

5.4.2.2 REFERENCE FOR POLYETHYLENE:

To reliably detect and quantify the presence of polyethylene microplastic particles in samples, the reference FTIR spectrum of PE serves as a crucial reference tool. This reference spectrum allows for the identification of the distinct

spectral fingerprints associated with PE, enabling accurate discrimination from other polymer types and sample matrix components. The characteristic PE absorbance bands are prominently located at 2917 cm^{-1} , 2852 cm^{-1} , and 718 cm^{-1} . The bands at 2917 cm^{-1} and 2852 cm^{-1} correspond to the asymmetric and symmetric C-H stretching vibrations, respectively, of the methylene ($-\text{CH}_2-$) groups present in the PE polymer backbone. Additionally, the band at 718 cm^{-1} is attributed to the rocking vibrations of these methylene groups. By systematically comparing the sample spectra with the PE reference and identifying the presence and relative intensities of these diagnostic bands, the occurrence and quantitative abundance of PE microplastics can be reliably established.

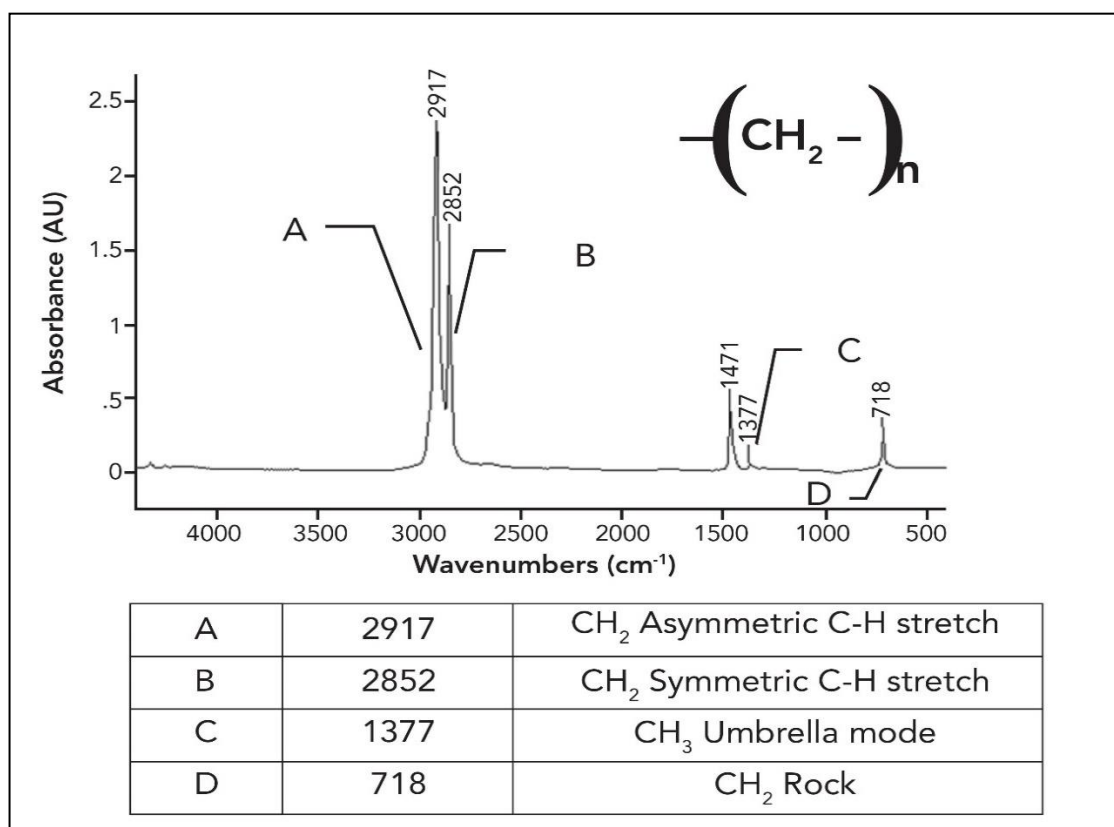


Fig 5.33: Reference FTIR spectrum of Polyethylene (D'Amelia et al., 2016)

5.4.2.3 M1 SAMPLE:

The FTIR characterization of the M1 sample has provided valuable insights into the chemical composition of the sample and the presence of polyethylene microplastics. The observed spectrum exhibits several prominent peaks that can be attributed to specific functional groups and molecular vibrations.

Notably, the peaks at around 2920 cm^{-1} and 2854 cm^{-1} correspond to the asymmetric and symmetric C-H stretching vibrations, respectively, of the methylene ($-\text{CH}_2-$) groups present in polyethylene. These peaks are characteristic markers for the identification of polyethylene. Additionally, the broad peak observed around 3284 cm^{-1} can be attributed to the O-H stretching vibrations of water and hydroxyl groups present in milk components, such as lactose and proteins. The peak at around 1639 cm^{-1} in the spectrum is likely due to the amide I band ($\text{C}=\text{O}$ stretching) from proteins present in milk, while the peak at around 1533 cm^{-1} corresponds to the amide II band ($\text{C}-\text{N}$ stretching) of milk proteins. These peaks are indicative of the presence of milk components in the sample.

Furthermore, the peak observed at around 718 cm^{-1} can be attributed to the rocking vibrations of the methylene ($-\text{CH}_2-$) groups in polyethylene, which is another characteristic peak for identifying this polymer. The presence of these peaks, along with the characteristic milk peaks (amide I, amide II, and lipid C-H stretching), suggests the presence of polyethylene microplastics or contamination in the milk sample under investigation.

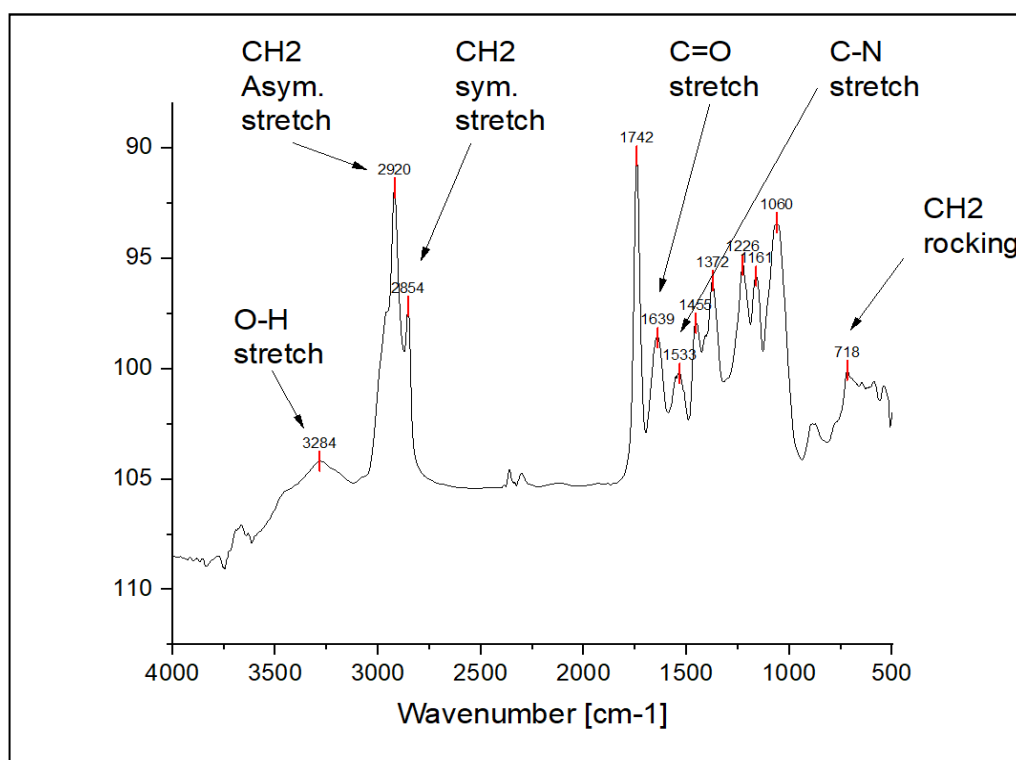


Fig 5.34: FTIR spectra of identified polymer in M1 sample

FTIR spectroscopy is a powerful technique for identifying the chemical composition of samples by analysing the characteristic vibrational modes of different functional groups and molecular structures. In this case, the combination of peaks associated with polyethylene and those related to milk components provides strong evidence for the presence of polyethylene microplastics in the milk sample.

5.4.2.4 M2 SAMPLE:

Notably, the peaks at around 2920 cm^{-1} and 2854 cm^{-1} correspond to the asymmetric and symmetric C-H stretching vibrations, respectively, of the methylene ($-\text{CH}_2-$) groups present in polyethylene. These peaks are characteristic markers for the identification of polyethylene. Additionally, the broad peak observed around 3330 cm^{-1} can be attributed to the O-H stretching vibrations of water and hydroxyl groups present in milk components, such as lactose and proteins. The peak at around 1639 cm^{-1} in the spectrum is likely due to the amide I band ($\text{C}=\text{O}$ stretching) from proteins present in milk. This peak is indicative of the presence of milk components in the sample.

Furthermore, the peak observed at around 718 cm^{-1} can be attributed to the rocking vibrations of the methylene ($-\text{CH}_2-$) groups in polyethylene, which is another characteristic peak for identifying this polymer.

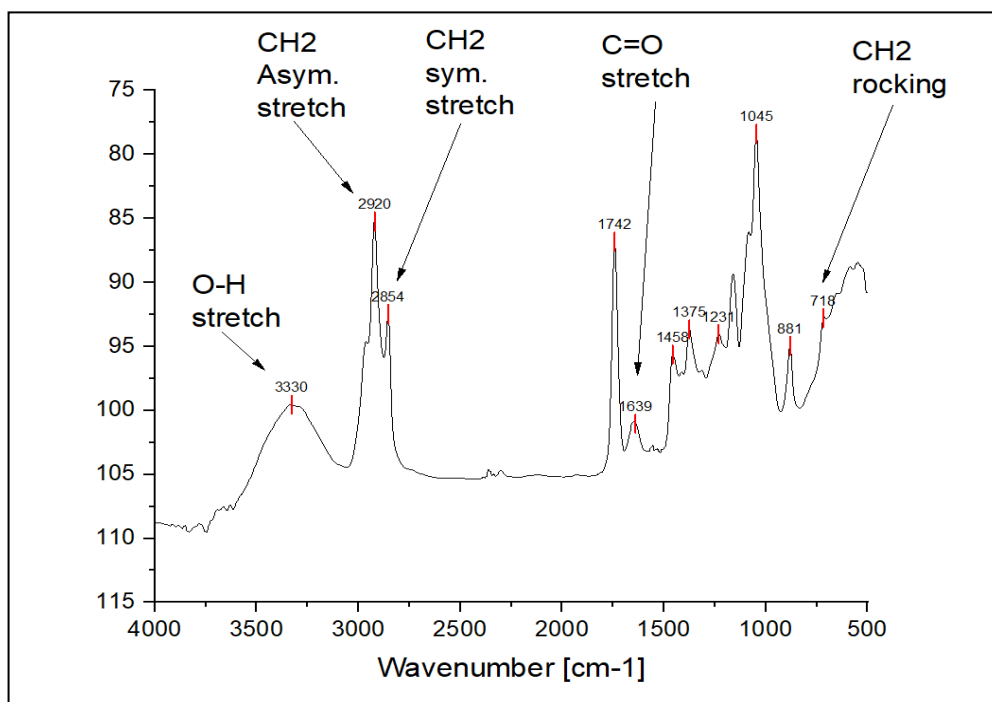


Fig 5.35: FTIR spectra of identified polymer in M2 sample

The presence of these peaks, along with the characteristic milk peaks (amide I and lipid C-H stretching), suggests the presence of polyethylene microplastics or contamination in the milk sample under investigation.

5.4.2.5 M3 SAMPLE:

Notably, the peaks at around 2917 cm^{-1} and 2849 cm^{-1} correspond to the asymmetric and symmetric C-H stretching vibrations, respectively, of the methylene ($-\text{CH}_2-$) groups present in polyethylene. These peaks are characteristic markers for the identification of polyethylene. Additionally, the broad peak observed around 3282 cm^{-1} can be attributed to the O-H stretching vibrations of water and hydroxyl groups present in milk components, such as lactose and proteins. The peak at around 1634 cm^{-1} in the spectrum is likely due to the amide I band ($\text{C}=\text{O}$ stretching) from proteins present in milk, while the peak at around 1538 cm^{-1} corresponds to the amide II band ($\text{C}-\text{N}$ stretching) of milk proteins. These peaks are indicative of the presence of milk components in the sample.

Furthermore, the peak observed at around 720 cm^{-1} can be attributed to the rocking vibrations of the methylene ($-\text{CH}_2-$) groups in polyethylene, which is another characteristic peak for identifying this polymer.

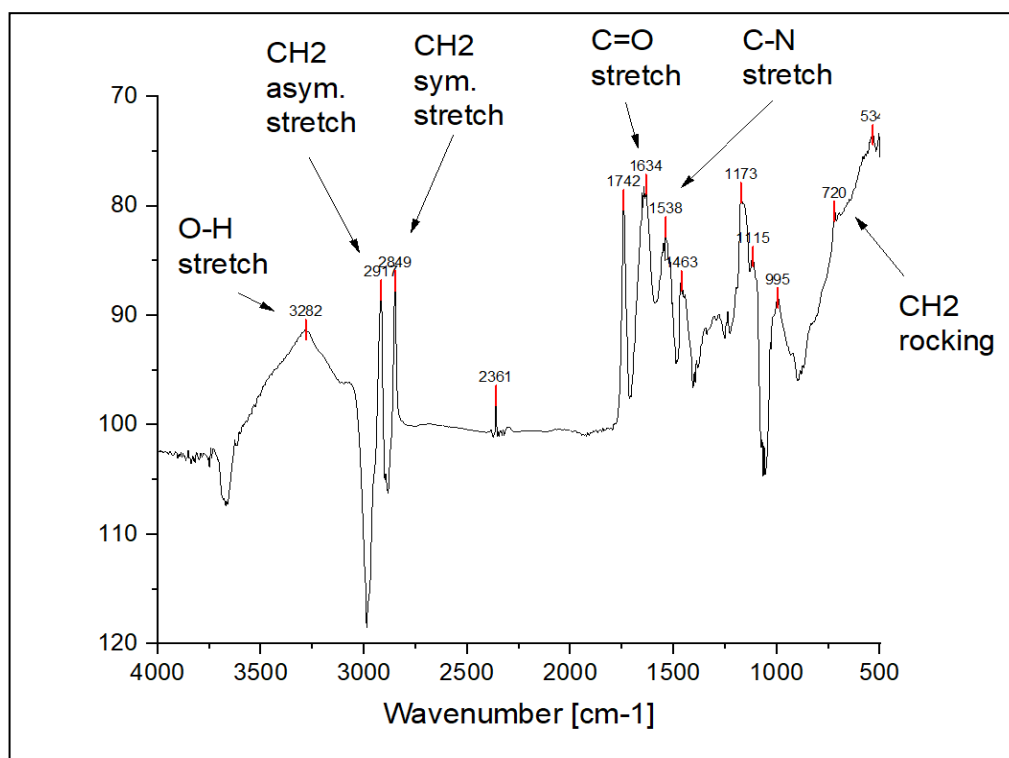


Fig 5.36: FTIR spectra of identified polymer in M3 sample

The presence of these peaks, along with the characteristic milk peaks (amide I, amide II, and lipid C-H stretching), suggests the presence of polyethylene microplastics or contamination in the milk sample under investigation.

5.4.2.6 M4 SAMPLE:

Notably, the peaks at around 2917 cm^{-1} and 2852 cm^{-1} correspond to the asymmetric and symmetric C-H stretching vibrations, respectively, of the methylene ($-\text{CH}_2-$) groups present in polyethylene. These peaks are characteristic markers for the identification of polyethylene. Additionally, the broad peak observed around 3282 cm^{-1} can be attributed to the O-H stretching vibrations of water and hydroxyl groups present in milk components, such as lactose and proteins. The peak at around 1634 cm^{-1} in the spectrum is likely due to the amide I band ($\text{C}=\text{O}$ stretching) from proteins present in milk, while the peak at around 1538 cm^{-1} corresponds to the amide II band ($\text{C}-\text{N}$ stretching) of milk proteins. These peaks are indicative of the presence of milk components in the sample.

Furthermore, the peak observed at around 718 cm^{-1} can be attributed to the rocking vibrations of the methylene ($-\text{CH}_2-$) groups in polyethylene, which is another characteristic peak for identifying this polymer.

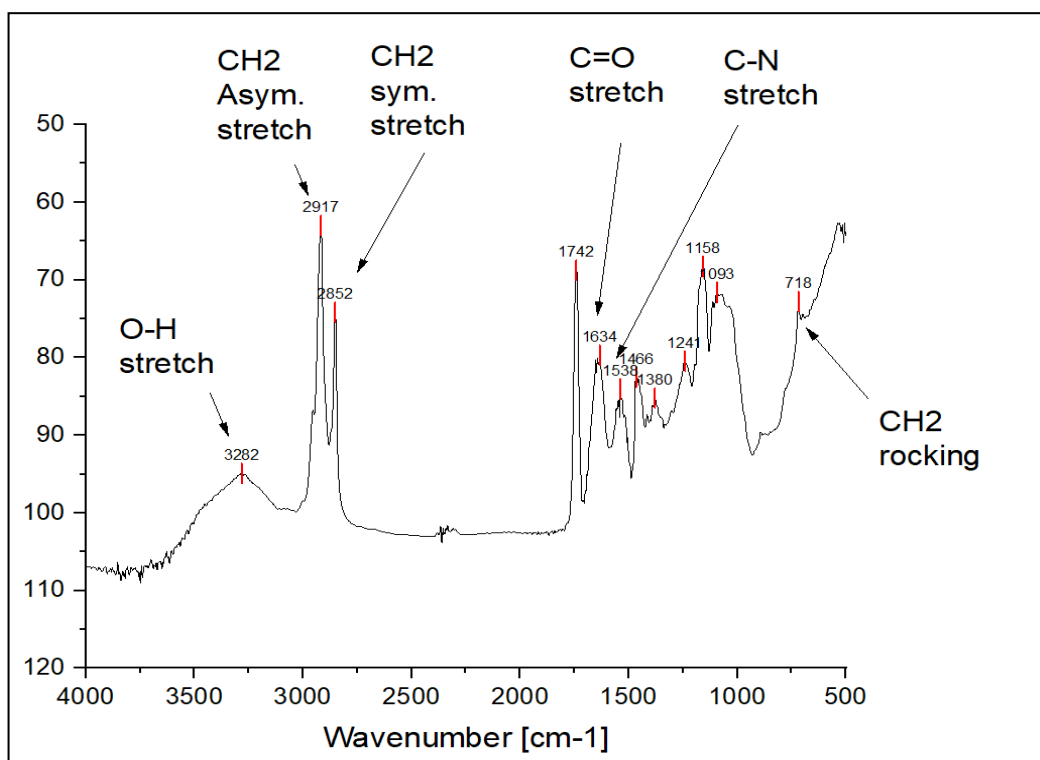


Fig 5.37: FTIR spectra of identified polymer in M4 sample

The presence of these peaks, along with the characteristic milk peaks (amide I, amide II, and lipid C-H stretching), suggests the presence of polyethylene microplastics or contamination in the milk sample under investigation.

5.5 CYTOTOXICITY OF MICROPLASTIC IN 3T3 CELL LINE:

5.5.1 ANALYSIS OF % OF CELL VIABILITY AND TOXICITY OF MICROPLASTICS IN 3T3 CELL LINE:

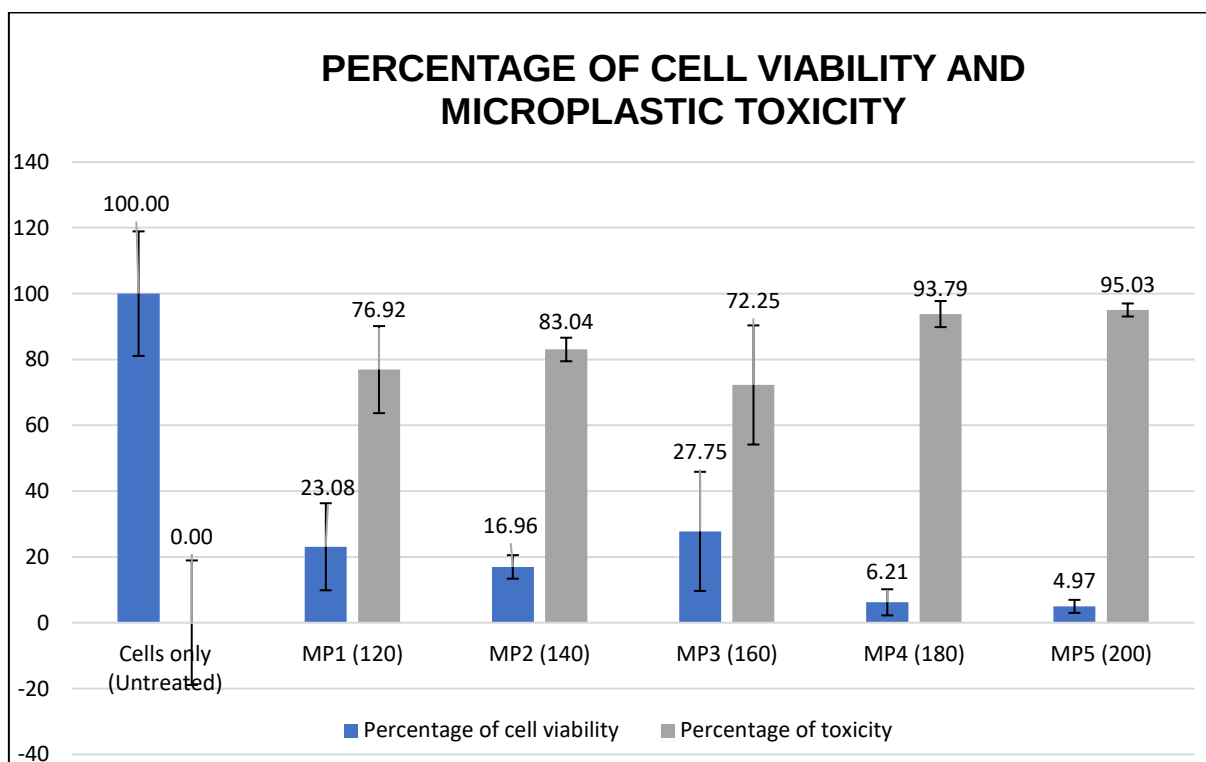


Fig 5.38: Graphical representation of % of cell viability and toxicity of microplastics in 3T3 cell line

From the figure, it can be stated that as the microplastic concentration increases from MP1 (120) to MP5 (200), there is a gradual decrease in cell viability, accompanied by an increase in microplastic toxicity. The highest cell viability is observed in the untreated cells (100%), while the lowest viability is seen with the MP5 (200) treatment (4.96%).

The results clearly demonstrate that microplastics exhibit cytotoxic effects on the 3T3 cell line, with higher concentrations leading to increased toxicity and reduced cell viability. The dose-dependent nature of the toxicity suggests that the microplastics interact with cellular components or processes in a concentration-

dependent manner, potentially causing oxidative stress and disruption of cellular functions. The observed cytotoxicity highlights the potential harmful effects of microplastic exposure on living organisms, particularly at higher concentrations.

5.5.2 STATISTICAL RESULT FOR MTT IN 3T3 CELL LINE:

Table 4.4: Anova: Single factor

SUMMARY				
Groups	Count	Sum	Average	Variance
Cells only	3	3.837	1.279	0.046033
MP + tween 20	3	0.436	0.145333	0.001381
Vehicle control	3	1.611	0.537	0.014932
Positive control	3	0.345	0.115	0.000081
Negative control	3	0.224	0.074667	8.33E-06
MP1 (120)	3	1.221	0.407	0.022477
MP2 (140)	3	1.013	0.337667	0.00164
MP3 (160)	3	1.38	0.46	0.042132
MP4 (180)	3	0.647	0.215667	0.002022
MP5 (200)	3	0.605	0.201667	0.000505

ANOVA						
Source of Variation	SS	DF	MS	F	P-value	F critical
Between Groups	3.357085	9	0.373009	28.42785	1.73E-09	2.392814
Within Groups	0.262425	20	0.013121			
Total	3.61951	29				

5.5.2.1 INTERPRETATION OF TABLE 4.4:

ANOVA (Analysis of Variance) results for the MTT assay, we can draw the following conclusions:

1. Significant differences exist: The ANOVA test compares the means of multiple groups to determine if there are statistically significant differences among them. In this case, the F-value of 28.42785 and the corresponding P-value of 1.73E-09 indicate that there are significant differences among the groups tested.
2. Concentration-dependent effect: The groups labelled "MP1" to "MP5" represent different concentrations of microplastics (120 µl to 200 µl). By observing the mean values for these groups, we can see a decreasing trend in cell viability as the microplastic concentration increases. This suggests a concentration-dependent cytotoxic effect of microplastics on the cell line tested.
3. Positive and negative controls: The "Positive control" group, likely treated with a known cytotoxic agent like DMSO, shows a low mean value (0.115), indicating reduced cell viability as expected. Conversely, the "Cells only" group (negative control) exhibits a high mean value (1.279), representing higher cell viability in the absence of any treatment.
4. Vehicle control: The "Vehicle control" group, which includes cells treated with the dispersant (Tween 20) used for suspending microplastics, shows a moderate mean value (0.537). This suggests that the dispersant itself may have some impact on cell viability, but to a lesser extent than microplastic treatments.
5. Comparison to controls: By comparing the mean values of the microplastic treatment groups (MP1 to MP5) with the "Cells only" and "Vehicle control" groups, you can assess the extent of cytotoxicity induced by microplastics compared to untreated cells and cells treated with the dispersant alone.

Given the F-value of 28.42785306 is significantly higher than the F critical value of 2.392814108, and the P-value is extremely low (1.73E-09, much less than the typical alpha level of 0.05), we can conclude that there are statistically significant differences among the means of the different groups tested in this MTT assay. This suggests that the treatments have significantly different effects on the dependent variable

Overall, the ANOVA results indicate that microplastics have a statistically significant effect on cell viability in the MTT assay, with higher concentrations leading to greater cytotoxicity.

CHAPTER-6

6. SUMMARY AND CONCLUSION:

This study successfully identified and characterised the presence of microplastics in milk and water samples through microscopic and spectroscopic techniques. Microplastics were observed in various colours and shapes, such as long fibres, fragments, and threads, with polyethylene being the predominant polymer detected. The cytotoxicity assays conducted on 3T3 cell line revealed an alarming increase in cellular toxicity with increasing concentrations of microplastics, highlighting potential adverse effects on human health and animals. Notably, based on the identification of a lower number of microplastics in the organic milk sample (M4) compared to other milk samples, we concluded that the presence of microplastics in milk could be attributed to the food and water consumed by cows, as well as potential contamination during handling and packaging processes, particularly with plastic covers. The reduced microplastic levels in organic milk suggest that organic farming practices and minimal processing may reduce microplastic contamination. While this study provides valuable insights, further research is crucial to elucidate the specific mechanisms underlying microplastic-induced cytotoxicity and to investigate the potential for microplastics to accumulate in various tissues and organs. Long-term exposure studies and epidemiological research are necessary to establish potential links between microplastic exposure and adverse health outcomes, considering factors such as age, gender, and pre-existing conditions. Addressing the growing challenge of microplastic pollution requires a multidisciplinary approach involving policymakers, industry, and researchers. Implementing effective waste management practices, exploring technological solutions for microplastic removal and degradation, and raising public awareness about the ubiquity of microplastics are crucial steps towards mitigating their release and accumulation in our environment and food systems. Ultimately, coordinated global efforts are essential to safeguard human health and environmental well-being from the far-reaching impacts of microplastic pollution.

Our project addresses the critical environmental and health concerns associated with microplastics contamination by identifying, formulating, and researching the technological problem through an extensive literature review and

interdisciplinary analysis (PO2). Complex investigations are conducted, involving experimental design, data analysis, and the synthesis of findings to draw valid conclusions about the presence, types, and associated risks of microplastics (PO4). Moreover, the project's direct contribution to addressing environmental sustainability concerns is evident, as it delves into the significant impacts of microplastics pollution on ecosystems and human health (PO7). By conceptualizing and implementing solutions to tackle microplastics contamination, including the assessment of potential health risks through cell line analysis, the project demonstrates a holistic approach towards addressing technical problems in both healthcare and environmental domains, thus underscoring its significance in advancing scientific understanding and fostering sustainable practices (PSO1).

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8. ANNEXURE:

PLAGIARISM REPORT:

11% SIMILARITY INDEX		
PRIMARY SOURCES		
1	link.springer.com Internet	166 words — 1%
2	www.researchgate.net Internet	148 words — 1%
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7	Campanale, Massarelli, Savino, Locaputo, Uricchio. "A Detailed Review Study on Potential Effects of Microplastics and Additives of Concern on Human Health", International Journal of Environmental Research and Public Health, 2020 Crossref	46 words — < 1%

PUBLICATION:

The project work has been published at the “11th International Conference on the Integration of Science and Technology for Sustainable Development-2024”

Isolation and Characterization of Microplastics from Different Sources of Water

Mahavishnou R¹, Sakthivel Jayaraj², Radhakrishnan Manikkam², Gopikrishnan Venugopal², Kasem Soyong³, Jayashree Shanmugam¹, Krupakar Parthasarathy^{2*}

¹Department of Biotechnology, Sathyabama Institute of Science and Technology, Chennai.

²Centre for Drug Discovery and Development, Sathyabama Institute of Science and Technology, Chennai.

³Department of Plant Production Technology, King Mongkut's Institute of Technology, Bangkok, Thailand.

*Corresponding Author Mail ID: pkrupakar.cddd@sathyabama.ac.in.

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Abstract Book: The 11th International Conference on the Integration of Science and Technology for Sustainable Development 2024 organized by Sathyabama Institute of Science and Technology, Tamil Nadu, India during 1-3, February 2024 (*ISBN: 978-93-83409-84-6*)

Microplastics refer to plastic particles that are smaller than 1mm and are now a global concern due to their presence in various ecosystems. While their impact on marine life and the environment is widely documented, their potential effects on human health are still being explored. In this study, we analyzed the presence of microplastics in water sources and their impact on human health due to accumulation in the body. Water samples from different sources were collected. Microplastic extraction was carried out by filtration method. The identification of microplastics was done using fluorescence microscopy. Identification and characterization of microplastics will be performed using FTIR. The material composition of microplastics will be identified using Micro-Raman spectroscopy. Further studies are being carried out and the findings will be discussed in detail during the presentation.

Keywords: Microplastics, Water sources, FTIR, Micro-Raman spectroscopy

REVIEW ARTICLE:

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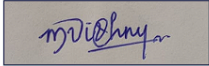
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Name of the student : Mahavishnou. R

Register Number : 40230023

Title of the Thesis : Identification and characterization of microplastics in milk and water samples and analyzing the effects of toxicity in cell lines

Internal Guide Name : Dr. S. Jayashree

External Guide Name : Dr. Krupakar Parthasarathy

PO / PSO / PEO / SDG	Justification
PSO 1	By conceptualizing and implementing solutions to tackle microplastics contamination, including the assessment of potential health risks through cell line analysis, the project demonstrates a holistic approach towards addressing technical problems in both healthcare and environmental domains, thus underscoring its significance in advancing scientific understanding and fostering sustainable practices
PO 2	Our project addresses the critical environmental and health concerns associated with microplastics contamination by identifying, formulating, and researching the technological problem through an extensive literature review and interdisciplinary analysis.
PO 4	Complex investigations are conducted, involving experimental design, data analysis, and the synthesis of findings to draw valid conclusions about the presence, types, and associated risks of microplastics
PO 7	The project's direct contribution to addressing environmental sustainability concerns is evident, as it delves into the significant impacts of microplastics pollution on ecosystems and human health

PEO 2	Our research topic on identifying and characterizing microplastics in milk and water samples, and analyzing their effects on cell lines, showcases our commitment as a motivated technologist. It highlights our research aptitude, lifelong learning through investigating an important issue, and multidisciplinary thinking by combining aspects of materials science, environmental science, and biology.
SDG 3	By understanding the presence and impact of microplastics in food and water sources, our work aims to uncover potential health risks, enabling measures to be taken to mitigate exposure and safeguard public health.
SDG 4	Our research promotes scientific literacy and awareness about the potential impacts of microplastics on our environment and health. Additionally, our research findings can inform educational curriculum and policies aimed at addressing this emerging issue.