UNIVERSITY OF SALFORD

SCHOOL OF SCIENCE ENGINEERING AND ENVIRONMENT

DETECTION AND IDENTIFICATION OF MICROPLASTICS IN FRESHWATER SAMPLES AND THEIR TOXICOLOGICAL IMPACT ON *DAPHNIA PULEX*, A MODEL ORGANISM FOR FRESHWATER.

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Table of Contents

LIST OF TABLES

LIST OF FIGURES

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DECLARATION

I HEREBY DECLARE THAT THE FOLLOWING WORK IS ENTIRELY MY OWN AND HAS NOT BEEN SUBMITTED, IN WHOLE OR IN PART, FOR ANY AWARD TO ANY OTHER ACADEMIC INSTITUTION.

LIST OF ABBREVIATIONS

ABS	Acrylonitrile Butadiene Styrene
ATR	Attenuated Total Reflection
BPA	Bisphenol A
DCS	Differential Centrifugal Sedimentation
FPA	Focal Plane Array
FTIR	Fourier Transform Infrared
GC	Gas Chromatography
GC–MS	Gas Chromatography-Mass Spectrometry
GPS	Geographical Positioning System
GPP	Global Plastic Production
LDPE	Low- Density Polyethylene
LLDPE	Linear Low- Density Polyethylene
MS	Mass Spectrometry
NGL	Natural Gas Liquid
MP	Microplastics
NP	Nanoplastics
NMR	Nanomagnetic Resonance
NTA	Nanoparticle Tracking Analysis
PA	Polyamide
PAI	Polyamide-imide
PBT	Polybutylene Terephthalate
PC	Polycarbonate
PE	Polyethylene
PEI	Polyetherimide
PET	Polyethylene Terephthalate

POM	Polyoxymethylene
PMMA	Poly(methyl methacrylate)
PP	Polypropylene
PPA	Polyphthalamide
PS	Polystyrene
PSU	Polysulfone
PTFE	Polytetrafluoroethylene
PVC	Polyvinyl Chloride
Pyr GC-MS	Pyrolysis Gas Chromatography Mass Spectrometry
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
μ-FTIR	Micro-Fourier Transform Infrared

ABSTRACT

Plastics have become an indispensable part of modern life due to their exceptional characteristics and versatility. Their use spans across various industries. At the beginning of plastic industry, the GPP was around 2 million tons, which reached approximately 368 million tons in 2019. However, these plastic products once consumed, are abandoned in environment, and enters waste stream, forming "Plastic Pollution". Despite being given much attention on its safe disposal and recycling' it is getting difficult to cope with plastic pollution. Particularly, when the abandoned microplastic objects undergo various degradation processes, they are converted into micro or nano plastics. Various studies suggested their increased toxicological effects with reduced plastic particle size. Given the importance of microplastic pollution, the present study investigated freshwater (from river Mersey) for detection and identification of microplastics using light microscopy, fluorescence microscopy and micro-Raman spectroscopy. Followed by this, experiments were conducted to evaluate the toxicological impact of microplastics (Low density poly-ethylene LDPE and Polyethylene terephthalate PET) on model invertebrate *Daphnia pulex* at physiological as well as molecular level. Finally, the study evaluated the potential of microwave assisted thermal treatment on degradation of PET microplastic particles under varied pH conditions. The results confirm the presence of microplastics in studied water samples but at very low concentration (0.01%). The toxicity of both studied microplastic types was seen in *Daphnia* in terms of reduced reproductive activity, irregular heart rate and high mortality (100% mortality observed for PE+Pb and <60% for PET+Fe and PE+Fe treatment groups) . The RNA expression analysis further validates the findings of bioassay. Upregulated expression of oxidative stress related genes (SOD, Vitellogenin and apolipoproteins), down regulation of development and reproductive pathway related genes indicates the compromised health condition

of *Daphnia* under microplastic exposure. The degradation of PET microplastic particles was observed under microwave assisted thermal treatment which reduces their potential to adsorb heavy metals and reduced toxicity impact.

CHAPTER 1

INTRODUCTION

1.1. Plastic; A Ubiquitous Material

Plastics are ubiquitous in nature. With the growing needs of the human population, their presence in environment have become inevitable. As the human population increases, demand for material resources rises. For instance, it needs more houses to build, more vehicles fortransportation, more food to eat and increased medical facilities. It would not be an exaggeration to say that our evolved lifestyle has become dependent on plastic products. Hence, one can find them used in building houses and buildings, manufacture of vehicles, space crafts, clothes, furniture, toys, utensils, household equipment's, packaging of food, agriculture sector and medical equipment Plastics possessing versatile chemical and physical properties make them the best choice to be used in every field of life.

The literal meaning of the word plastic (derived from as Greek words 'Plastikos' meaning 'fit for moulding' and 'Plastos' meaning 'moulded') explains well its property that makes it beneficial over other materials such as steel or iron. Due to diverse chemical composition, plastics possess many characteristics that increase its usability on large scale. For instance, plastic products being low-cost, versatile, durable and very lightweight in comparison withmetal ones, are in high public demand and it will continue to grow in the coming years. Total production of plastics around the world reached 359 Mt in 2018 (Garside, 2019), which was just 245 Mt in 2008. European production slowed to 61.8 Mt in 2018 down from

64.4 Mt in 2017 (Klemes *et al*., 2020). China being the largest producer of plastic products, contributed 30% of total plastic produced in 2018 whereas 17% came from Europe. In Europe, 9.4 Mt of plastic used waste was collected for recycling, as consumers and the industry paid more attention to plastic disposal and end-use as reported in 2019 by Plastics Europe (2020) (Klemes *et al*.,2020).

Consumption of plastics has been increased in every aspect of life. From making toys to construction of buildings and even automobiles, plastics serve user friendly purposes and thus making its use inevitable. For example, very light weight plastics are crucial for space exploration (Naser and Chehab, 2020). However, probably the major and the most substantial use is packaging. Plastics are the perfect materials to be used for packaging goods. Irreplaceable use of plastics is also seen in the health care sector. Plastics are used for single-use medical tools (Chen *et al*., 2020), packaging and even for some medical surgeryand transplants. However, the increase in global plastic consumption has increased the waste stream diversity and made it extremely difficult to manage. A large proportion of plastic products are single use and thus enter the waste stream adding up to the plastic debris. This makes plastic pollution a newly emerged environmental problem to be faced by human population. Once enter the waste stream plastics debris undergo long-term tearing and wearing but it does not undergo complete environmental degradation. Remnants of plastics could be present at micro or nano scales for over centuries (Chamas *et al*.,2020).

1.2. Chemical Nature of Plastics

Natural resources that include petroleum, natural gas and coal are generally considered asraw materials for production of plastics. Small biomolecules of these raw materials are used as basic building blocks for plastic production and are termed as 'monomers. For plastic production, the raw materials are gone through complex chemical process called 'polymerization' as it results information of long chains (polymers) of same of different monomer units. During the process, polymers are restructured both chemically and physically and the resulting material is capable of moulding, casting, extruding and blown to almost any imaginable forms, from minute submicron fibers to foams, films, and large objects with lifelong durability. Hence, Plastics are diverse group of synthetic or semi synthetic complex polymers with very high molecular mass (10,000 to 500,000).

1.2.1. Plastic Manufacturing Process

As mentioned above, plastics are basically derived from natural organic compounds (organic compounds are made of carbon and hydrogen mainly) such as petroleum, natural gas, and mainly crude oil. However, the total oil consumption for plastic production remains very low that is 4-6 % of global oil consumption. (Herve Millet et al., 2018).

Plastic synthesis began with the distillation of crude oil and then separation of heavy and lighter crude oil fractions. Each fraction is a mixture of hydrocarbon chains, which differ based on size and structure of basic

hydrocarbon molecules. One such fraction is "naphtha" (see figure 1.1 for process of plastic production). Chemical modification such as cracking of this crude oil fraction "naphtha" produces many chemically different molecules such as ethylene, styrene, propylene which serve as monomers for plastic production (Plastics Oceans, *Cleaner Oceans Foundation Limited*).

The combining of polymers leads to production of long chain polymers and hence the process is called polymerization. As illustrated in figure 1.1, the polymerization process involves two main chemicalprocesses i-e polyaddition (simply addition of monomers one after other) and polycondensation (addition of monomers through chemical bond which results in loss of water molecule). Properties of resulting plastic polymer depends on type of monomer used, its structure and formulation (Crawford & Martin., 2020).

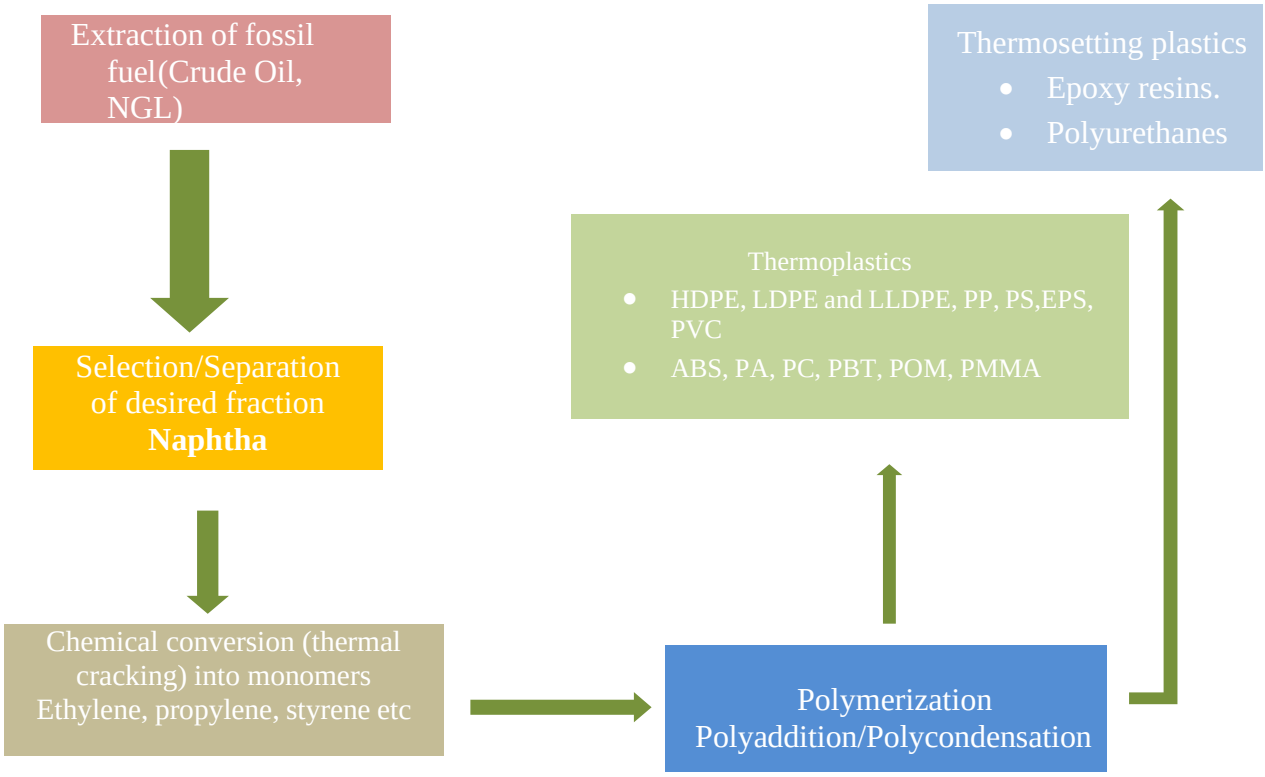


Figure 1.1: Process of plastic production from raw material fossil fuel (Crude Oil, Natural gas liquids NGL; Thermosetting plastics HDPE=high density polyethylene, LDPE=Low density polyethylene, PP=Polypropylene, PS=Polystyrene, EPS=Expanded Polystyrene, PVC=Polyvinyl chloride, ABS=Acrylonitrile butadiene styrene, PA= Polyamide, PC=Polycarbonate, PBT=Polybutylene terephthalate, POM= Polyoxymethylene, PMMA= Poly(methyl methacrylate)).

1.2.2. Revolution in Plastic Industry

Modern plastics have been revolutionized and are now serving much more as compared to the plastics produced in 1950s. To give them limitless useful properties, pristine plastic polymers are often mixed with functional chemical additives that alter the polymer properties and produce diverse forms of modern plastics. Most used additives include functional additives, colorants, fillers, and reinforcements. Among all these, functional additives are used the

largest groups of additives to be used in modern plastic industry. Additives majorly comprise of plasticizers that can make upto 70 % (w/w) of plastic polymer. Plasticizers help improve the flexibility and durability of plastics, flame retardants that make them able to be used safely in electronics and antioxidants, which delay oxidation when exposed to UV (Crawford & Martin., 2020). Other functional additives that are used in relatively lower amounts include heat stabilizers, lubricant, slip agents and biocides. To give them coloring or attractive fluorescent effects, organic (molecules that have carbon and hydrogen atoms as basic elements) or inorganic pigments are used as colorant additives. Filler additives such as mica, calcium carbonate or talc are used normally to reduce the material cost whereas reinforcement additives are used to increase tensile strength and reduce shrinkage (Crawford & Martin., 2020). With the use of these additives, huge catalogue of plastic products is produced that possess enormously diverse range of properties.

1.3. Types of Plastics

Plastics are broadly classified into two main types 1) Thermoplastics and 2) Thermosetting plastics.

1.3.1. Thermoplastics

These are the plastics which can be melted upon heating and hardened when cooled. These mechanically recyclable types of plastics account for approximately 80% of total plastic demand (Biron. M, 2016). Properties of

thermoplastics may vary based on their chemical structure and performance capability and so are sub categorized as i) Standard Plastics which are most widely used ones and account for major portion of global thermoplastic demand (Biron. M, 2016). It includes polyolefins (which comprise of all types of polyethylene (High density polyethylene, Low density polyethylene and Linear low-density polyethylene) and polypropylene), polyvinyl chloride (PVC), polystyrene (PS), expanded polystyrene (EPS) and polyethylene terephthalate (PET) (Rhodes, 2018) ii) Engineering Plastics, these are relatively high-performance plastics particularly in terms of heat resistance, chemical resistance, mechanical strength, and fire retardants. These plastics are mostly used in the building and construction industry and account for around 10% of total thermoplastic demand. Examples are ABS=Acrylonitrile butadiene styrene, PA= Polyamide, PC=Polycarbonate, PBT=Polybutylene terephthalate, POM= Polyoxymethylene, PMMA= Poly (methyl methacrylate iii) High Performance Plastics; these plastics possess very have mechanical and chemical performance capability and thus provide exceptional end-use applications. Examples include fluoropolymers Polytetrafluroethylene, PTFE, Polyimide PI, Polyamide-imide PAI, Polyether imide PEI, Polysulphone PSU and Polyphthalamide PPA.

1.3.2. Thermosetting Plastics

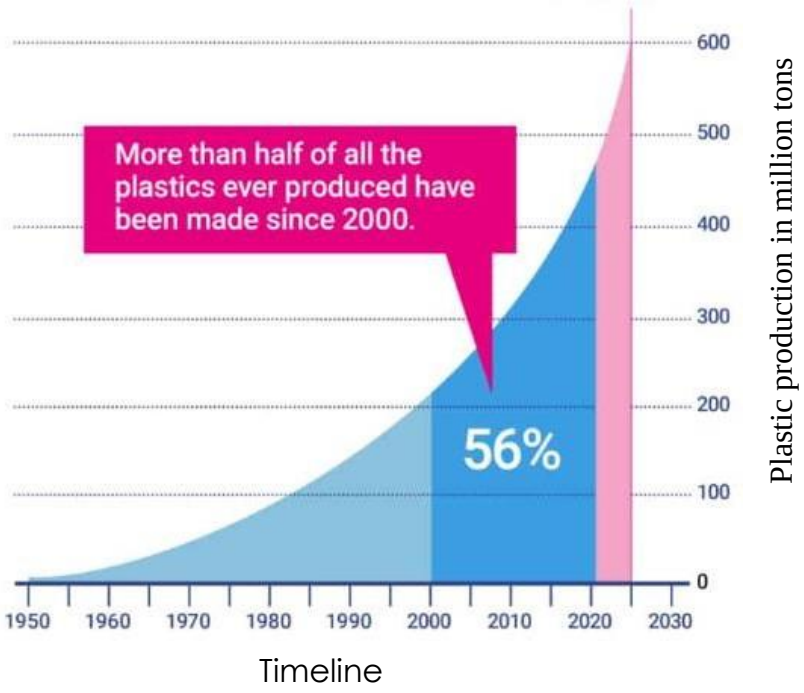
These are the types of plastics which when heated undergo permanent chemical changes and are deformed permanently. Hence, these are non-

recyclable plastics. Majorly this type of plastics are used in gas and water pipelines, automotive parts, medical equipment, construction machinery parts, signage, storage boxes, electrical plugs and casings, kitchen appliances and toys. Main thermosetting plastics include i) epoxy resins; commonly used as protective lining or coating material in various sectors such as food packaging, furniture, toys. They are also used in special paints to protect surfaces such as ships, wind turbines and oil rigs against harsh weather. They help prevent metal corrosion and improve food shelf life, ii) polyurethane; these are the plastics composed of carbamates (Urethane) monomers. Being thermally stable, these plastics are extensively used as insulated buildingpanels, furniture, car seats, refrigerators.

1.4. Global Plastic Production

Plastic use has become indispensable in human life. With the increasing demand in almost every industrial sector, global plastic production is exploding. In 1950s when plastic industry just begun, the Global Plastic production (GPP) was around 2 million tons, which reached approximately 368 million tons in 2019 (Kumar *et al*., 2021). More than half of the total plastic produced since 1950s, has been manufactured after 2000. Asia being more populated is acknowledged as largest contributor (51% of GPP) followed by the North American Free Trade Agreement (NAFTA) countries (Canada, Mexico, and the United States; 19%), Europe (16%), the Middle East and Africa (7%), Latin America (4%), and the Commonwealth of Independent

States (Azerbaijan, Armenia, Belarus, Georgia, and other;3%). (Plastics Europe. Plastics—The Facts 2020, Statista. Production of Plastics Worldwide from 1950 to 2019 (in Million Metric Tons). 2021). Geyer et al (2017) estimated that plastics litter in landfills and natural ecosystems will reach 12 billion tons by 2050.



(Plastic soup foundation, 2022)

Figure 1.2: Global Plastic Production n Trendline from 1950 till 2020; estimated production in next 10 years; x-axis: time in years; y-axis: Plastic production in million tonnes

According to recent (2022) estimates given by "Plastic Soup Foundation" a major portion of total plastic produced is consumed for packaging purposes, that accounts for approximately 40%. Of total GPP. This is followed by Building and construction (20.4%), Automotive (9.6%), Electrical and electronics (6.2%),

Household/Leisure & sports (4.1%),Agriculture (3.4) and other (16.7%).

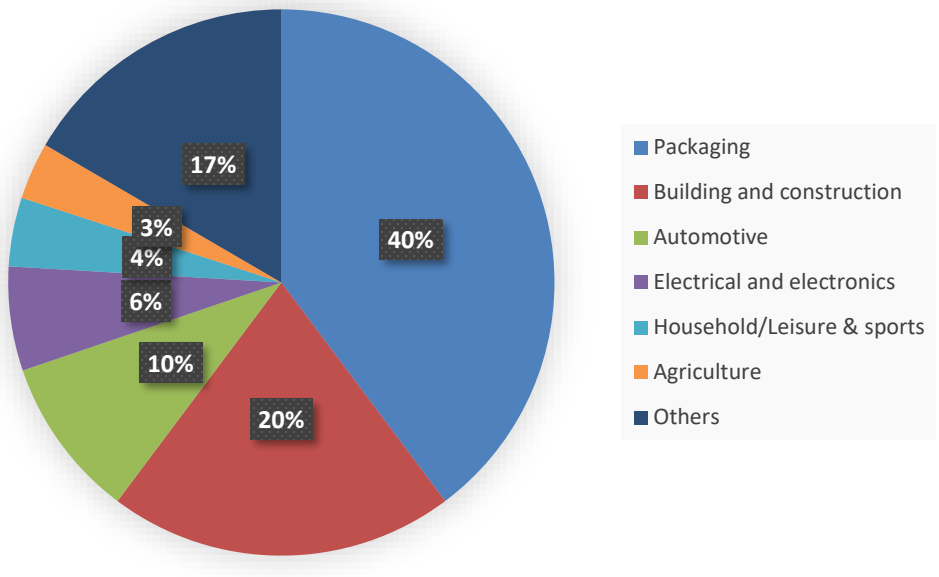


Figure 1.3: Plastic resource demand distribution by industrial sector; presented in percentage.

1.5. Plastic as Waste

Overproduction of plastics has created havoc because after consumption, the plastic enter waste stream. It becomes troublesome due to inadequate recycling, inappropriate disposal to landfills and unsafe release to the environment. Because most plastics are non- biodegradable, they linger in the environment for decades undergoing tearing and weathering and changing from macro to micro forms. Studies on investigating the fate of plastic waste have gained due attention in the last few years due to its underlying health impact. From 1950 till 2015, 6.3 billion tonnes of plastic waste that includes both primary and secondary (recycled) waste was generated

(Science Daily, July 2017). Out of this, 79 % is either being stored in landfills or released into the

environment whereas only 9 % being recycled or incinerated (12%) (Rhodes, 2020, Gayer etal 2017).

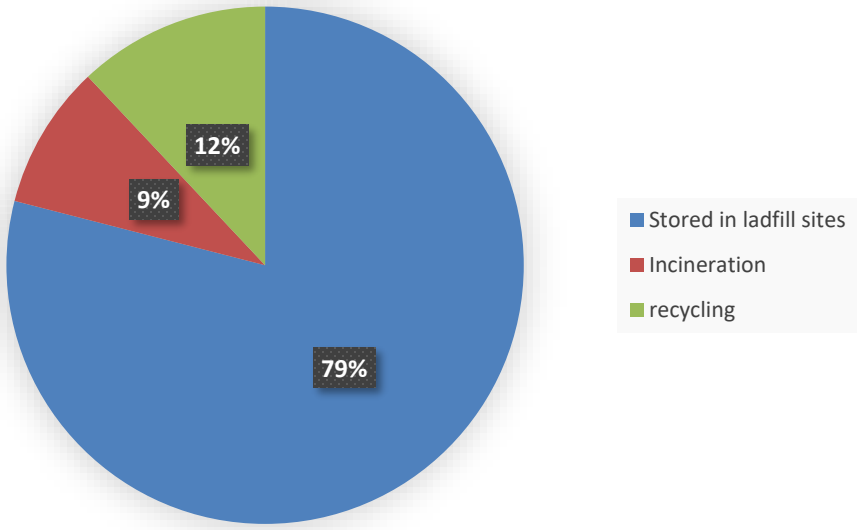


Figure 1.4: Plastic waste disposal methods

Landfilling is an organized method for disposal of biodegradable as well as non- biodegradable waste in a designated terrestrial site or landfill (Nanda, 2020). Most of the countries use landfilling technology as a conventional method for waste disposal and is considered convenient over other waste disposal procedures such as incineration (Nanda, 2020). However, disposal of plastic waste to landfills renders the designated land unavailable for other productive purposes such as agriculture (Zhang *et al*., 2004). Despite this, the long- term non-degradation of plastic waste in the landfill sites serves as source of secondary pollutants which include volatile organic species (VOCs),

such as benzene, toluene, xylenes, ethylbenzene and trimethyl benzene isomers, either as gaseous components, or *via* leachate

(Xu *et al*., 2011), along with various other substances, including Bisphenol A (BPA) (Tsuchida *et al*., 2011), with the risk of groundwater contamination. BPA is considered a very toxic chemical and has been recognized as an endocrine disrupter (Tsuchida *et al*., 2011). Plastic waste also enters water bodies on a large scale. Oceans serve as big sink for secondary waste. From a study by Orb Media, it is claimed that (Tyree & Morrison, 2017) 'an estimated 1 million tons of these tiny microfibers are discharged into wastewater every year, where more than half evade treatment and escape into the environment', which indicatesthat up to 0.5 Mt or so might enter the ocean.

1.6. Plastics as Major Environmental Pollutant

Unmanaged plastic waste in different environmental strata is referred to as "Plastic Pollution". It is now recognized as an important long lasting anthropogenic change to the surface of earth. The suspected underlying impact of plastic pollution has gained due scientific attention in recent years (Rhodes, 2017). Investigations are revealing strong evidence for direct or indirect toxicological effects of plastic pollution on both marine and freshwater biota, which will be discussed in following section (Galloway *et al*., 2017; Lusher *et al*., 2017).

1.7. Impact of Plastic Pollution

Plastic pollution has become a global public health concern for the last few decades. Researchers are giving more attention to revealing its underlying impact on different environmental components (Jambeck *et al*., 2015). The

potential implications of plastic pollution have been reported on land and water (Rochman *et al*., 2016; Ng *et al*., 2018) 60% of the total plastic mass produced, generates plastic waste that is accumulated in landfills or dispersed to waterbodies and other environment globally (Geyer, Jambeck, and Law, 2017). Plastic pollution has been reported to have direct impact on croplands due to extensive use of plastics in agriculture sector such as use of mulch films (defined and explained in section 1.10) and biosolids (Duis & Coors, 2016; Steinmetz *et al*., 2016). In recent decades, use of plastic mulch films in croplands have reached 1.5 Mt annually worldwide, 60%–80% of which is used on China's croplands (Espi, Salmeron, Fontecha, Garcia and Real, 2006; Yang *et al*., 2015). The uncollected mulch films start accumulating in croplands and thus the accumulation of these mulch film residues damages the physical structure of soil and alters soil hydrology (Liu *et al*., 2014). These physical soil changes help to explain the reduction in root weight, which in turn affect the nutrient uptake of crops and ultimately, result in the reduction of cropyield (Zhang *et al*., 2020). Reduction in soil microbial biomass as well as enzyme activity has also been reported to have direct relation with increasing film residue content (Wang *et al*., 2016). Moreover, the contamination of soil by plastic additives such as phthalates, have also been associated with the use of plastic film mulch (Gao *et al* 2018; Wang *et al*., 2013). Plastic additives that exhibit endocrine-disrupting, mutagenic, and carcinogenic properties are a cause for concern (Zhang *et al*., 2020). These plastic additives are consumed by plants, move along the food web and

ultimately end up in top level consumers such as humans (Fu and Du, 2011; Wang *et al*., 2015).

Another important negative impact of increasing plastic waste is the landfill leachate. Although landfilling technology is serving as a conventional waste management procedure, its long-term effects are inevitable (Nanda *et al*., 2020). Anaerobic conditions are developed inside a landfill due to several layers of soil on the disposed of organic matter, which results in anaerobic digestion and landfill gas generation (Tałałaj *et al*., 2019). The chemicals produced in landfill leachate are very toxic. Because this landfill leachate has potential to contaminate groundwater leading to biomagnification, it is classified as hazardous (Wiszniowski *et al*., 2006; Pasalari *et al*., 2018). Thermal, chemical and biological reactions occurring at landfill site also led to release of toxic landfill gases. Landfill gas is acknowledged as a contributor to global warming issue due to its two main constituents such as CH_4 and CO_2. It has been reported that the potential of CH_4 in global warming is 25 times more than that of CO_2 and has a lifetime of 12 years in the atmosphere (Nanda *et al*. 2016). Moreover, Landfills are also ranked as the third-largest contributor of CH_4 in the USA after the fossil fuel industry and agriculture, especially livestock farming (USEPA, 2020). The high concentration of combustible CH_4 in landfills gases also has potential risks of accidentalfires and explosions.

The impact of plastic pollution is attributed to its physiochemical properties of plastic pollutants. Most important characteristic is the size of plastic particle.

Therefore, it is very important to discuss the size-based classification of plastic pollutants.

1.8. Size based Classification of Plastic Pollutants

Plastics have been classified in different ways, based on their size, chemical properties, or polymer composition. However, classification based on size is considered very important, especially when considered as major environmental pollutants. Many studies including those of Roch et al., 2021, Woods et al., 2018 documented an inverse relationship between the toxicity of plastics and particle size and size-dependent ingestion of plastics by biota. Based on this, Woods et al. (2018) concluded that size should be considered as an important criterion in future studies of plastics as environmental pollutants.

Generally based on their size, plastics and the plastic pollutants are categorized into following five types (Cauwenberghe *et al.,* 2015; Gigault *et al.,* 2018; Frias and Nash, 2019).

- Macro Plastics: Plastic particles greater than 25mm in any of the direction are generally referred to as macro plastics. These include include plastic chairs, shoes, parts of vehicles – cars, ships and planes – buoys, footballs, plastic shopping bags, and many other commonly used items. For example, Ghost nets are becoming major plastic pollutant of aquatic biosphere. It is a fishing net that has been lost or abandoned, so that it drifts along with the ocean tides and currents, and traps sea creatures and additional macro debris,

eventually becoming fully laden, mainly with other plastic objects. Such ghost nets have been reported to accumulate to masses of perhaps 6 tones, by when they are too large, and too heavy to be recovered from the ocean (Hammer, 2012). The greatest densities of the world's plastic pollution have accumulated around water fronts and urban centers in the Northern Hemisphere, although such depositions may also collect off the coasts, and wash onto the beaches of particularislands as a result of the directional flow of currents (gyres) which transport the debris.

- Meso Plastics: This category includes plastic particles between 5 – 25 mm. These are mostly larger fragments of big plastic objects. Bottle caps fall into this category.
- Microplastics: Plastic particles ranging between 5mm – 1µm are termed as Microplastics. Particles that are <0.001 mm (1 µm). The size definition is not very clear and there is some debate which size is nanoplastic, some studies suggest <0.1 µm (Klaine et al., 2012), others suggest <20 µm (Wagner et al., 2014).

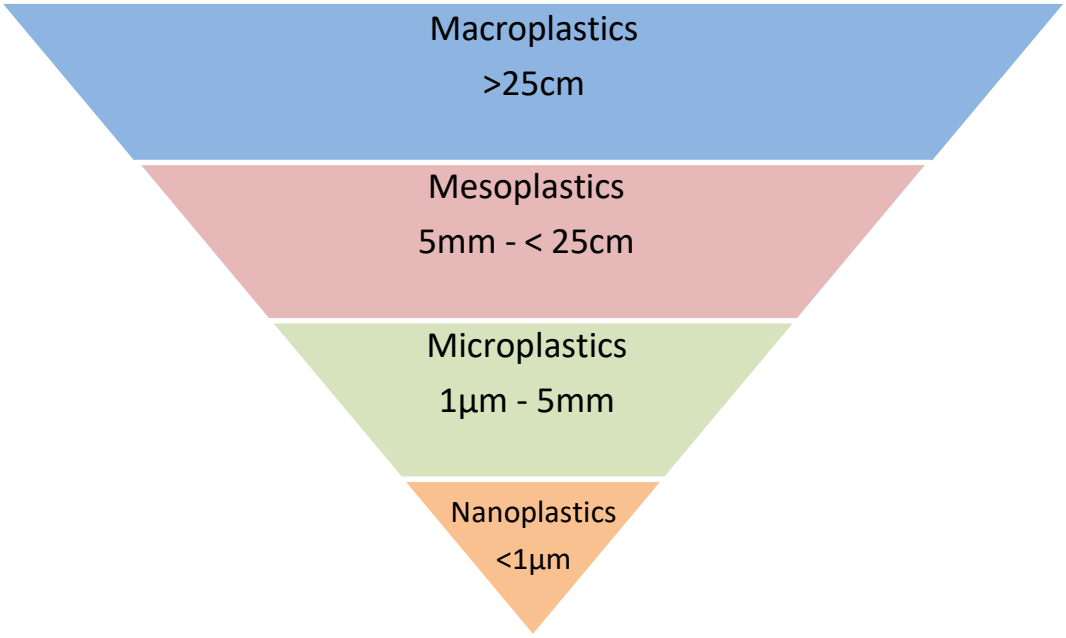


Figure 1.5: Chronological representation of types of plastic pollutants based on their size.

As mentioned above, the toxicological impact of plastic pollutants increases with decrease in size. Hence, the present study is focused on Micro and Nano sized plastic particles as potential environmental pollutants.

1.9. Microplastics

Microplastics are very small plastic particles with size less than 5mm in its longest dimension. These particles could be regular or irregular shape or surface, that depends on their source of origin. The lower size limit set for microplastics is 1µm. (Frias and Nash,2019).

1.9.1. Types of Microplastics

Microplastics are further classified based on their source of production as primary and secondary. Primary microplastics are plastic particles which are originally synthesized at the size in which they are encountered in the environment such as microbeads, scrubbers used in exfoliator handwash or face cleansers or many other industries (Rochman *et al*., 2015). Whereas secondary microplastics are formed when larger plastic objects are tarnished into small fragments by mechanical or physical action, photodegradation, and a variety of other methods (Driedger et al., 2015), and hence, plastic pollution that originally linked to macrodebris, can be broken down into a larger number of smaller (microdebris) particles (Rehse, 2018; Andrady, 2011). Most microplastics in the marine environment fallinto this secondary category (Eriksen, 2014; Cozar, 2014).

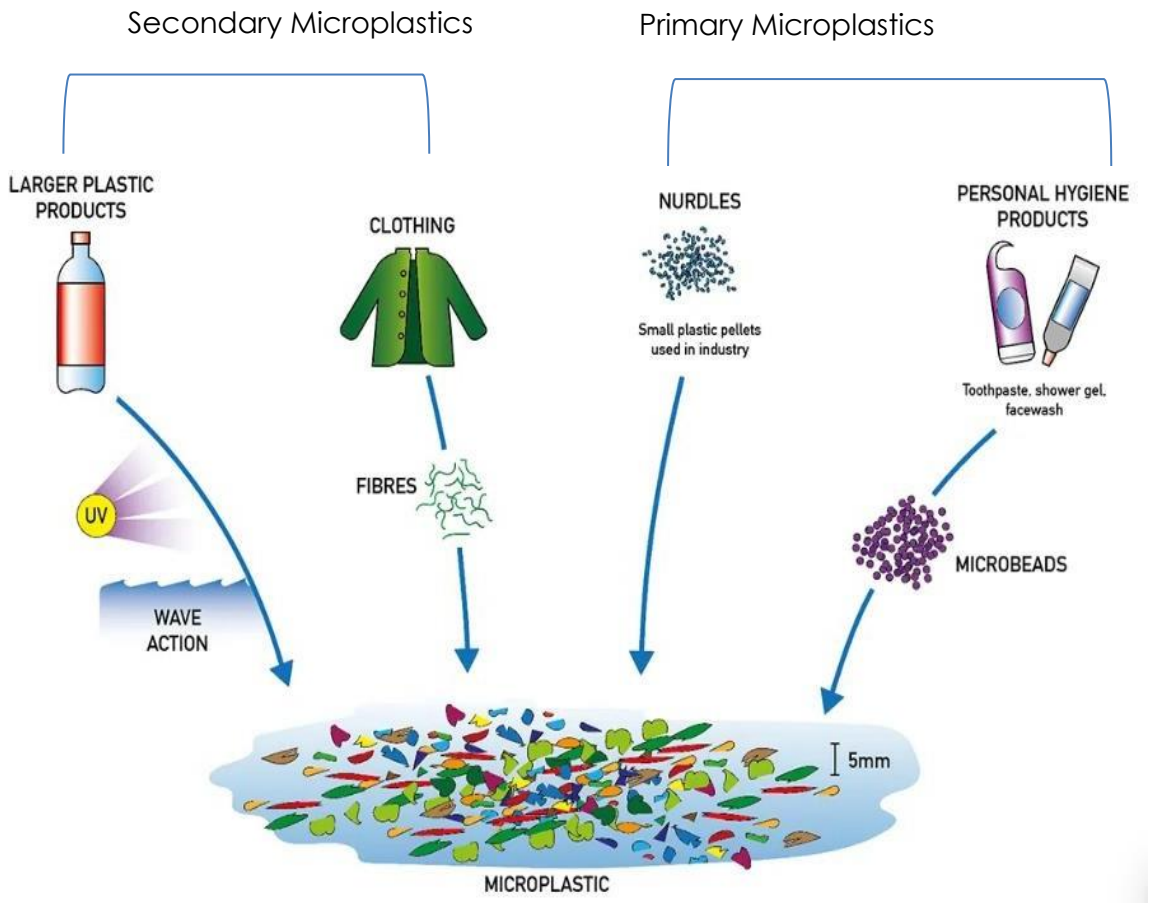


Figure 1.6: Microplastic classification based on their source of production; Secondry microplasticsgenerated from tearing and wearing of bigger plastic objects; Primary microplastics manufacturedin micro size forms. (encountered.com, "where do plastics come from")

Microplastics in environment exhibit potential to act as vectors for transport of important organic pollutants (Rochman, 2015). The vector potential of microplastics could be categorized into three groups (Syberg *et al.*, 2015) 1) environmental vector in which microplastics transport the adhered molecules to the environment, 2) organism vectors in which the toxic particles attached to microplastic are transported to the living organisms and finally ,3) cellular

vectors in which the microplastics transport the particles into the cells.

1.9.2. Physical Properties of Microplastic Particle

Size:

Like every other chemical particle, microplastic particles are attributed to physical as well as chemical properties. Considering the importance of particle size in toxicological impact of micro/nanoplastics, researchers are focused on evaluating potential of several techniques for detection of micro/nanoplastic model particles as well as particles in different environmental samples such as water, sediments, organs or tissues. Several techniques have been explored in this regard. For example, the recent processing techniques allow the researcher to identify particles as small as 10 µm (µ-FTIR (micro-Fourier transform infrared spectroscopy with a focal plane array FPA detector) or downto 1-2 µm (µ- RAMAN) (Chakraborty *et al*., 2023). Three size classes could be made to record microplastic data, which reflect current sampling and processing practices namely: 1 ≤ 100 µm; 100 ≤ 350 µm and from 350 µm to ≤5 mm, as this allows an easy comparision of identified particles for research purposes (Frias and Nash, 2019).

Types:

According to a criterion established by Gago *et al.*, (2018) microplastics could be classifiedinto eight categories mentioned as

- Pellet
- Fragment

- Fiber
- Film
- Rope and filaments
- Microbeads (perfect spheres)
- Sponge/foam
- Rubber

Most frequently obtained types of microplastics in environment are fibers, fragments, films, foams, microbeads and pellets. However, the abundance of secondary microplastic types is higher (fragments, fibers, and foams) than the primary microplastics (microbeads and pellets) (Cozar, 2014; Eriksen, 2014). Identification of specific type of plastic particle in studied samples could give clue to what sources they have been originated from. Such as, if a sample contain more fibers, it could be assumed that the sample might have content coming from garments industrial waste or domestic waste that includes wastewater from washing washings.

1.9.3. Mechanism of Secondary Microplastics Production

Mechanically weathered plastics become susceptible to additional weathering methods.

such as photo-degradation, thermal-degradation or biodegradation resulting in production of secondary microplastics. Three basic degradation processes are involved in production of secondary microplastics i-e photodegradation, thermal degradation and biodegradation (Bond *et al*., 2018). Among these, photodegradation is considered as the most important method of plastic degradation. In this method the unsaturated parts of the plastic polymer absorb UV light and produce polymer radicals. This is followed by addition of oxygen and removal of hydrogen in the monomers thus leading to cleavage of polymer chain (Feldman, 2002; Singh and Sharma, 2008; Gewert *et al*., 2015). Thermal degradation occurs in the same way except that instead of UV, heat initiates polymer reactions (Singh and Sharma, 2008; Gardette *et al*., 2013). Thermal degradation of plastics is a commercial degradation process, which rarely occurs in the environment (Mattsson *et al*., 2015, Shen *et al*., 2019). Biodegradation also plays role in formation of secondary micro plastics but unlike other environmental pollutants, microorganisms have shown to have limited activity on plastic polymers (Tokiwa *et al*., 2009; Ng *et al.*, 2018,). The extent of formation of secondary polymers from their parent entities varies greatly. For example, in polypropylene (PP) every other carbon atom bonding to methyl group is more prone to chemical attack as compared to polyethylene bonding to hydrogen (Gewert *et al*., 2015). polystyrene is susceptible to abiotic degradation because the phenyl ring can be excited by UV light. The excitation energy is transferred to nearby carbon and

enhance the formation of polymer radicals (Yousif and Haddad, 2013). The bond dissociation energies are in the order of PE (402 kJ mol^{-1} at UV light of 300 nm) > PS (377 kJ mol^{-1} at UV light of 318 nm) > PP(322 kJ mol^{-1} at UV light of 370 nm), indicating that PP is moreproneto UV degradation (Song *et al*., 2017).

1.10. Nanoplastics

Nanoplastics are nanosized plastic particles produced from fragmentation of larger plastic particles. Thus, nanoplastics are defined as any synthetic or semisynthetic plastic polymer with size of <100nm in at least one of its dimensions (Klaine *et al*., 2012). Because nanoplastics are generated as result of spontaneous weathering or abrasion of larger plastic particles, they are highly diverse in physical properties and heterogeneous in chemical composition (Gigault *et al*., 2016, Lambert and Wagner, 2016, Ter Halle *et al*., 2017). Moreover, nanoplastics due to their polydisperse nature tends to form aggregated with other natural or anthropogenic materials (Hüffer *et al*., 2017). It is reported earlier that when plastic particles reach nanoscale, they can interact with microbes such as bacteria or phytoplanktons. This interaction alters the buoyancy of nanoplastic particles either in a positive and negative fashion (Long et al., 2015; Lagarde *et al*., 2016). Despite this, the significant Brownian motion of nanoplastic particles prevent them to sediment and thus keep floating in water bodies (Hassan *et al.,* 2015).

1.11. Sources of Micro/Nanoplastics in Environment

Although plastic pollution has raised concerns to global health organizations and gained suspicion of scientific community (Jambeck *et al*., 2015), It seems that the short-term benefits of plastics override the long-term serious health issues and so their use persists.

The key source of micro/nanoplastics entering the agriculture system includes plastic mulch films, municipal waste (e.g. municipal solid waste, compost), biosolids (sewage sludge and anaerobic digestate), plastic-coated fertilizers and atmospheric deposition (McCormick *et al*., 2014; Nizzetto *et al*., 2016; Andrés Rodríguez-Seijo, 2018; Blasing and Amelung, 2018; Liu *et al*., 2018) .Of these, agricultural films and compost application are probably the most important ones (Blasing and Amelung, 2018, Hurley and Nizzetto, 2018). The use of plastic mulch films has become an important technique which helps promote agricultural production in different regions across the world. Water and nutrient resource have been found to be enhanced explicitly. Moreover, these Plastic mulch films provide thermal insulation as well as helps in early planting and/or harvest cropping (Yin *et al*.,2014; Gao *et al*., 2019; Liu *et al*., 2019). Hence, plastic mulching technique may lessen soil erosion and help reduce the crop disease burden. Further, it allows the more efficient use ofpesticides (Yan *et al*., 2010, Ruíz-Machuca *et al*., 2015). Based on the above- mentioned advantages, the use of plastic mulching has been promoted on large scale by various agriculture extension agencies and other

related industries in order to promote greater food security, sustainable food production and improve livelihoods (Liu *et al*., 2014, Yan *et al*., 2014, Steinmetz *et al*., 2016). But unfortunately, the underlying health concerns due to large scale use of plastic mulching techniques have been neglected. There are many issues that need to be addressed seriously in this regard. One of the major issues is that these films are very thin (ca. 8–50 µm thick) thus making it nearly impossible to extract them from the soil at the end of growing season (Liu *et al*., 2013) (Figure 1.1 shows abandoned mulch films). The extreme concern of this mulch films is that assisted by ploughing, UV irradiation and biodegradation. These residual mulches gradually fragments making a continuum of macro, micro and nano plastics in soil (Ramos *et al*., 2015, Steinmetz *et al*., 2016).

Figure 1.7: Large amount of plastic mulching film residues in farmland in China. A and B show recycled film residues, while C and D show residual film in agricultural soils. The photos were taken in Gansu Province by Yan Changrong in 2018.

The second important source of addition of micro/ nanoplastics to land is the recycling ofbiosolids. Traditionally, recycling of biosolids has been of great help in agriculture systems since it provides a way of closing the nutrient cycling loop on the other hand it replenishesthe organic matter in cropping soil (Singh and Agrawal, 2008; Sullivan, 2015). But recentinvestigations are suggesting the recycling of biosolids may encompass substantial amounts of plastic pollution (Gatidou *et al*., 2019). Typically, between 70 and 99% of the microplastics present in domestic wastewater are recovered in the sludge fraction during water treatment (Carr *et al*., 2016) leading to microplastic concentrations in sludge of 10^3– 10^5particles kg^{-1}. Consequently, large amounts of microplastics

will accumulate in the soil, particularly after repeated applications of sewage sludge to agricultural land (Andrés Rodríguez-Seijo, 2018, Nizzetto *et al*., 2016).

Municipal solid waste landfills may also represent point sources of microplastic pollution affecting the underlying soil and groundwater (Andrés Rodríguez-Seijo, 2018; Duis and Coors, 2016). In the leachate fraction, microplastic particles range from 100 to 1000 µm insize with a concentration of 1–25 particles l^{-1} (He *et al*., 2019).

1.12. Migration and Dispersal of Micro/nanoplastics in Environment

Though microplastics were discovered as early as in the 1970s (Carpenter & Smith, 1972)), scientific research strengthened only after time-series data highlighted increasing microplastic contamination of Atlantic waters and microplastic ingestion by marine biota (http://litterbase.awi.de/interaction_detail; date accessed: 17 November 2018).. Since then, MP has been identified in all marine realms from beaches to the deep seafloor and in all oceans and seasworldwide. (Bergmann *et al*., 2017). Plastic in this size range is of particular concern because it can be taken up by a wider range of biota and be propagated in food web (Cole *et al*., 2013; Leslie *et al*., 2017). Microplastics are more easily to be ingested and difficult tobe egested by biota (Cole and Galloway, 2015, Fueser *et al*., 2019, Jeong *et al*., 2016, Jin *et al*., 2018). Besides, the change in particle sizes can affect the distribution of microplastics in the tissues of organisms (Lu *et al*.,

2016, Su *et al*., 2019). Lu *et al*. (2016) suggested that the 5-μm polystyrene micro particle was accumulated in the gill, liver and gut of zebrafish, while the 20-μm was only accumulated in the gill and gut. Hence the distribution of micro/nano plastics in the environment have gained due attention.

Microplastics can also be transported and dispersed by soil animals and livestock, either through attaching to the outside of the organism or through transfer from ingestion and defecation (Cao *et al*., 2017; Rillig *et al*., 2017). Microplastics can also be transferred to aquatic ecosystems by surface runoff (Blasing and Amelung, 2018; Brodhagen *et al*., 2015; Hurley and Nizzetto, 2018; Kyrikou and Briassoulis, 2007; Steinmetz *et al*., 2016). The migration of microplastics through surface runoff is related to the particle size and density of the microplastic. The bulk density of common plastics typically varies from 910 to 970 kg m^{-3} depending on the nature of the material. Therefore, plastics without much soil mineral contamination (densityof2650 kg m^{-3}) readily float. In addition, the migration is easier for smaller particles as there is less likelihood of physical trapping in the soil matrix or surface vegetation (Nizzetto *et al*., 2016; Li *et al*., 2019; Li *et al*., 2019). Additionally, theshape, type, and surface characteristics of microplastics are also important factors which are likely to affect their migration in soils. Thus, it is vital to further study the weathering process, adsorption capacity, and migration of microplastics, especially those with a particle size <1 mm (Zhou *et al*., 2018). These studies will not only be beneficial to understanding the distribution and migration of microplastics in marine and terrestrial ecosystems, but also provide an important reference for protection and governance of marine, freshwater and terrestrial ecosystems.

1.13. Adsorption of Organic Pollutants and Heavy metals on Micro/Nanoplastics

Organic pollutants are long lived, toxic organic compounds that have been found in different compartments of environment i-e air, soil, water (Jiamo Fu *et al*., 2003) The most commonly encountered persistent organic pollutants are organochlorine pesticides, such as DDT, industrial chemicals, polychlorinated biphenyls (PCB) as well as unintentional by-products of many industrial processes, especially polychlorinated dibenzo-p-dioxins (PCDD) and dibenzofurans (PCDF), commonly known as dioxins (World Health Organization WHO). Being toxic, they have a negative health impact on humans as well as other living organisms and have been of global public health concern. Relatively recent issue of microplastics pollution seems to have aggravated organic pollutants problem. Due to their small size and hydrophobic nature, organic pollutants have been widely associated with microplastics (Alimi *et al*., 2018; Barletta *et al*., 2019). Different studies provide evidence for presence of organic pollutants on microplastics present in the environment as well as the microplastic particles ingested by different living organisms (Rhode *et al*., 2018). Some of the important microplastics found in the environment include polystyrene, polypropylene and polyethylene. These microplastics have been reported to have some importantorganic pollutants on their surface such as environmental endocrine disruptors, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), and 2,2-bis(p-chlorophenyl)-1,1,1-

trichloroethane (DDTs) (Wang *et al*., 2020). Toxicity, bioaccumulation, and degradation of organic pollutants has been reported to be greatly influenced by microplastics due to kinetics and equilibrium of the sorption/desorption of these compounds on/from Microplastics (Koelmans *et al*., 2013; Koelmans *et al*., 2016). Very small size, large specific surface, and the hydrophobic nature of microplastics are the characteristics that favor their interaction with organic pollutants in environment. (Andrady, 2011; Hartmann *et al*., 2017; Alimi *et al*., 2018; Ceccarini *et al*., 2018; de Souza Machado *et al*., 2018; Wang *et al*., 2018a). León *et al*., 2018 reported in their investigations that these organic pollutants absorbed on microplastics when exposed to fresh or sea water could be absorbed within 24 hours and thus increasing their concentration in marine ecosystem (León *et al*., 2018).

Heavy metals are also very important environmental pollutants. Heavy metals in aquatic environments are found due to the natural process of rock weathering and are also introduced by human activities. Research suggests that anthropogenic actions have disrupted the natural biogeochemical cycle of heavy metals (Haghnazar *et al*., 2022). Currently, the occurrence of heavy metals in aquatic surroundings is primarily linked to human activities (Guan *et al*., 2018). Typically, sediments contain a significantly larger amount of heavy metals that originate from human sources (Jiang *et al*., 2021). River sediments are particularly susceptible to contamination by heavy metals. Heavy metals from human activities such as industrial waste are generally

found in higher levels and can pose toxic risks to aquatic organisms (Yi *et al.,* 2016). After being ingested by aquatic organisms, heavy metals enter the food chain (Yi *et al.,* 2016) and subsequently pose potential threats to human well-being (Emenike *et al*., 2020).

Given the significance of heavy metals as environmental pollutants, and potential of microplastics to act as vectors, this study aimed at investigating the combined effect of microplastics and heavy metal (Pb^{2+} and Fe^{2+}).

1.14. Bioaccumulation

The bioaccumulation of organic pollutants is increased when they are adsorbed by micro or nano plastics. This increase further depends on the size of the micro or nanoplastic particle. Investigations on the role of micro or nano plastics on increased bioaccumulation of organic pollutants revealed that they serve as carrier molecules and help transfer of organic pollutants up in food web. (Besseling *et al*., 2013; Ma *et al*., 2016; Wardrop *etal*.,2016; Chen *et al*., 2017; Granby *et al*., 2018; Qu *et al*., 2018; Qu *et al*., 2019; Zhao *et al*., 2020). For example, research done by Ma *et al*., 2016 concludes that, 50 nmpolystyrene nanoplastic particles significantly enhanced the bioaccumulation of phenanthrene in the body of *Daphnia magna* with 40-times higher adsorption capacity while the bioaccumulation made by 10-µm PS particles was relatively insignificant.

1.15. Degradation of Micro/Nanoplastics In Environment

Due to the durable nature of plastic particles, they are considered as

persistent organic pollutants in the environment. However, like every other particle in nature, plastic particles are also prone to several degradation processes although it takes several hundreds of years to undergo complete degradation. Such degradation processes include photodegradation (UV catalyzed oxidation), hydrolysis, biodegradation and mechanical degradation (Gewert *et al*., 2015; Zambrano *et al*., 2020). Weathering of plastic particles in aquatic and soil environment by photodegradation is a vital process (Gewert *et al*., 2015). Generally, the degradation process begins with discoloration and surface cracking of the plastic particlesand thus cause embrittlement by increasing particle surface area (Bond *et al*., 2018). Further in combination with other mechanical factors such as winds and waves ultimately lead to mineralization of plastic particles by microbes to CO_2 (Gewert *et al*., 2015; Da Costa*et al*., 2018). Plastic particles posses' chemicals additives that were added to the initial product for desirable properties. These additives are not covalently bound to the plastic particles and thus are more vulnerable to the degradation process that results in leaching of the chemical additives (Wang *et al*., 2018).

Biodegradation is a very important degradation process for plastic particles. However, in the environment this degradation process is coupled with mechanical or physiochemical degradation. Though the plastic particles are not particularly susceptible to microbial attack as other biodegradable products (Rujnic-Sokele and Pilipovic, 2017) still they provide good

environmental niche for microbial colonization by supporting growth and serving as a carbon source. Several microbes have been identified that are involved in plastic particle degradation such as bacterial pure cultures as well as, fungi and biofilms. A significant decrease in weight of plastic particles has been observed (Devi *et al*., 2015).

1.15.1. Bacterial Mediated Biodegradation of Micro/Nanoplastic Particles

Bacteria are notorious for their extraordinary abundance in every compartment of the environment. Besides their abundance, bacteria gained due scientific attention due to its potential ability to degrade various environmental pollutants (Bakir *et al*., 2014). In recent years, some bacterial strains have been reported to degrade micro plastic particles. An investigation by Auta *et al*., 2018, used two pure bacterial cultures collected from mangrove sediment and used for micro plastic particle degradation. Auta also assayed the ability of two isolates, *Bacillus cereus* and *Bacillus gottheilii*, to degrade different types of Microplastics. The results interestingly revealed that microbial activity causes several pores on the surface of treated micro plastic particles. This concludes that the pure bacterial cultures do have potential to adhere, colonize and damage microplastic particles. However, studies suggest that the weight loss of microplastic particles because of bacterial degradation is very low such as 0-15% and hence plastic particles could be said as poorly biodegradable particles (Yuan *et al*., 2020).

On the contrary to this, there are some studies which suggest that pure bacterial cultures produce some toxic compounds that inhibit its growth (Dobretsov *et al*., 2013). These findings are further supported by evidence that encourages the use of combination of bacterial strain for degradation of microplastics. The combination of bacterial strains called bacterial consortia serves better for degradation purposes because it creates stable bacterial community by inhibit the toxicity of compounds released by individual strains. In this scenario, the toxic chemicals released by one strain could be used as substrate for other bacteria thus nullifying its effect on bacterial community and increasing biodegradation potential (Singh and Wahid, 2015). Moreover, a study by Tsiota *et al*. 2018 demonstrated that two different consortia show different potential for degradation of plastic particles. However, the degradation of microplastic polymers through bacterial consortia is a complex process and so far the respective studies have shown potential only under controlled conditions so extensive research is required to develop an efficient bacterial consortium in open environment in order to have maximum degradation potential consequently reducing microplastic pollution.

1.15.2. Fungi Mediated Degradation of Micro/Nanoplastics

Like bacteria, fungi have also been of great interest to scientific community because of its wide distribution as well beneficial environmental potential. Recent studies have explored that fungi can adhere to and interact with

microplastic particles (Mitik-Dineva *et al*., 2009). With microplastic particles, fungi can promote making chemical bonds such as carboxyl, carbonyl or ester bonds that results in decrease of hydrophobicity of microplastic particle. Moreover, fungi may encourage the transformation and circulation of different substances (Chen et al., 2016). In one such study by Sangeetha Devi (Devi *et al*., 2015) two fungi species were investigated for their MP degradation potential. First species investigated was *Aspergillus tubingensis* VRKPT1 collected from marine coastal area and was tested on high density polyethylene microplastic particles with 30 days exposure time. Results showed that there was significant decrease in weight of plastic particle and used it as carbon source. Another species investigated was *Aspergillus flavus* VRKPT2 which demonstrated same results on high density polyethylene particles (Devi *et al*., 2015). An investigation by

Russell *et al*., 2011 demonstrated that fungi have potential to degrade different microplastics such as polyurethane (PUR). The research identified a serine hydrolase enzyme which was responsible for the degradation of polyurethane microplastic particles, suggesting that the enzymes secreted by this fungus have potential for microplastic biodegradation. At present, various high-density polyethylene (HDPE)- degrading fungal strains have been isolated from PE waste dumped in marine coastal areas and screened under *in vitro* conditions. These results demonstrated the excellent ability of these fungal strains to degrade Microplastics under *in vitro* conditions. Thus, future studies could involve the use of metagenomic mining techniques to increase the discovery rate of fungal-mediated MP degradation (Paco *et al*., 2017).

1.16. Factors Affecting Microbe-Mediated Degradation of Micro/Nanoplastics

The degradation of microplastics by microorganisms depends on various factors (Yuan *et al*., 2020). Broadly classifying, they could be of two main categories. On first place there

are factors related to the growth of microorganisms, secondly factors related to the characteristics of microplastics and the external environment. The characteristics of microplastics affecting biodegradation include both physical and chemical properties such as density, molecular weight, crystallinity, functional groups and substituents present in its structure (Shah *et*

al., 2008). Environmental factors that mainly affect microplastics degradation include light (UV), heat, humidity, and the presence of chemicals (Yuan *et al*.,2020). Environmental factors assist the degradation activity in two aspects. On one hand they affect the metabolism and growth of microorganisms and on other hand they promote the aging and damage of microplastic particle by external oxidation thus accelerating the degradation and utilization of microplastic by microorganisms (Krueger *et al*., 2015).

1.17. General Mechanism of Microbe-Mediated Microplastic Degradation

As explained above, Plastics are long chain organic polymers, so the reactions involved in their degradation are much like other non-plastic polymers. Microplastic polymers are large molecules relative to the pore size of microbial cellular membrane. Thus, microplastic polymers must be depolymerized so that they can be absorbed by microbes forbiodegradation and mineralization (Yuan *et al*., 2020). Before mineralization polymers are converted to monomers. Microorganisms have been reported to possess certain enzymes that help in degradation of polymers. The process use for degradation of microplastics by microorganisms is through hydrolysis. As an example, a serine hydrolase enzyme which was responsible for the degradation of polyurethane microplastic particles, was identified in fungus species suggesting that the enzymes secreted by it have potential for microplastic biodegradation (Russell et al., 2011). However, microplastic

hydrolysis is contrasted with oxidative degradation, which can transform both hydrolyzable and nonhydrolyzable polymers. During the hydrolysis process, the enzyme from microbes binds to the microplastic particles, followed by catalyses hydrolytic cleavage. The important enzyme involved in biodegradation is depolymerases (either intracellular or extracellular) found in different microorganisms. The process of microbial breakdown of microplastics into smaller chemical moieties (e.g., monomers, ddimers, and oligomers,) that are small enough to pass through semipermeable membranes is referred as depolymerization. These short-chain molecules are then mineralized into end products (e.g., CO_2, H_2O or CH_4) through a process called mineralization, and these products can then be utilized as carbon and energy sources (Gu, 2003; J. Yuan *et al*., 2020). Many studies involving the separation and identification of enzymes in microorganisms have been performed in recent years (Lau et al., 2009). Yoshida et al. (Shosuke Yoshida *et al*., 2016) isolated a novel bacterium, Ideonella sakaiensis 201-F6, that can use PET as its major energy and carbon source. When grown on PET, this strain produces two enzymes (ISF6-4831 and ISF6-0224) that are capable of hydrolyzing PET and the reaction intermediate, mono(2-hydroxyethyl) terephthalic acid.

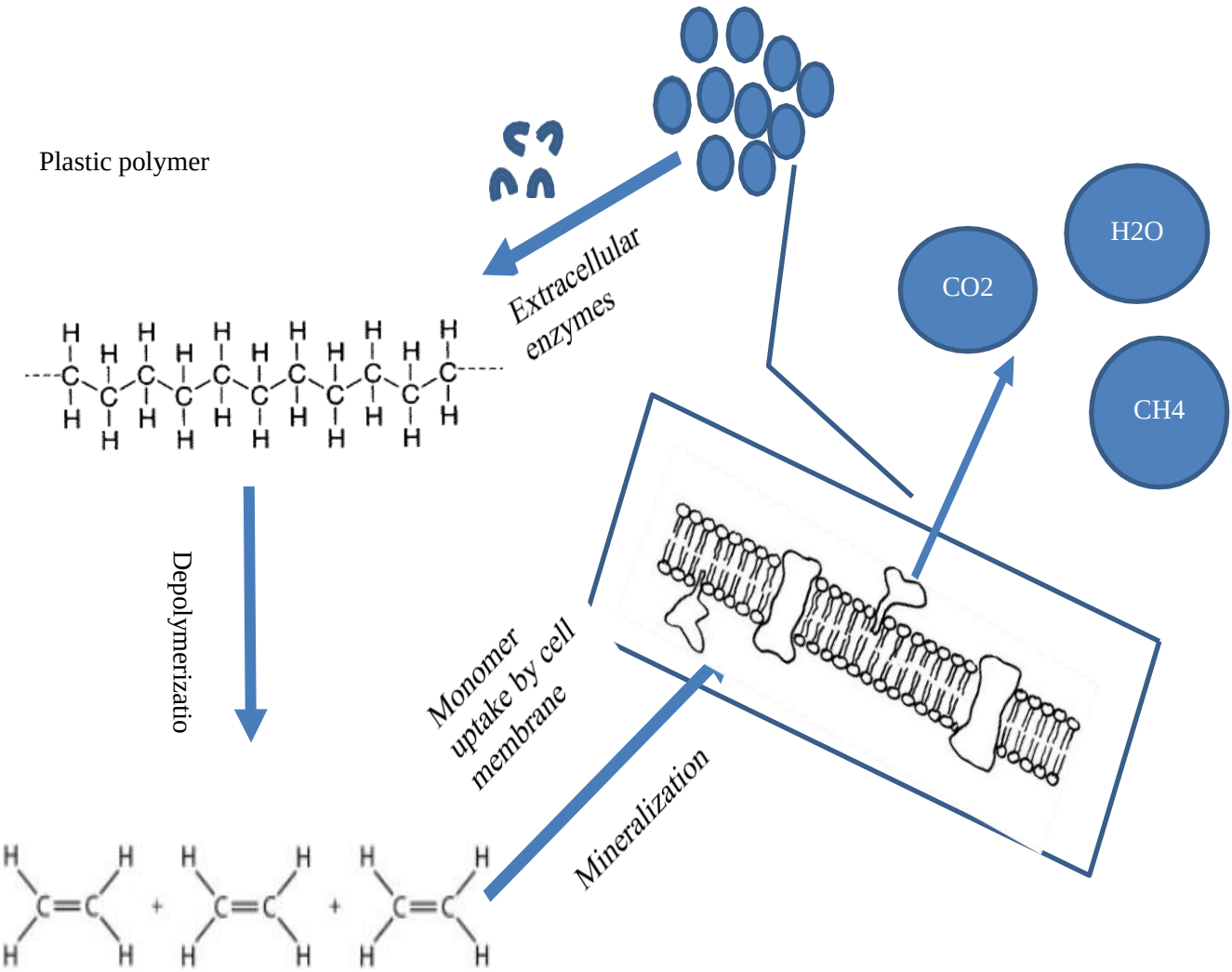


Figure 1.8: Schematic representation of Microbe mediated plastic degradation process.

Although microbial degradation seems a promising method to reduce microplastic pollution, it will take significant research effort to implement such technology on large scale and harvest its benefits.

1.18. Microplastic Pollution in UK

The assessment of microplastic and nanoplastic pollution in freshwater ecosystem has not given due attention in UK either. Only a small number of investigations until now have reported the contamination of freshwater system with microplastics although they have previously been predicted to be equally pervasive (Eerkes-Medrano *et al*., 2015). In the UK, most of the investigations were restricted to rivers (Vaughan *et al*., 2017). However, the first study reporting the abundance and distribution of microplastics was conducted in central Birmingham, UK in the surface sediments of an urban lake (Vaughan *et al*., 2017).The research findings demonstrate that maximum concentrations reached 25–30 particles per 100 g dried sediment (equivalent to low hundreds kg^{-1}). Hortan *et al*., 2017 assessed the sediments of tributaries of the River Thames, UK for microplastic particle abundance and quantification method. Four target sites were identified to be contaminated with microplastic particles. One site had significantly higher numbers of microplastics than other sites, average 66 particles 100 g^{-1}, 91% of which were fragments (Hortan *et al*., 2017). The most common polymers identified in this investigation included polypropylene, polyester, and poly aryl sulphone. The quantification technique used in most of the studies was FTIR. These

findings provide evidence of growing burden for microplastic ubiquity in all environments and the dire need of more advanced techniques for their accurate quantification.

Thus, given the wide-ranging damaging effects of microplastics it is essential that we should identify and quantify environmental contamination levels.

1.19. Toxicological Effects of Micro/Nanoplastics

The physical and chemical properties of plastics are enhanced on micro and nanometric scale. The very small size of micro/nanoplastic particle increases the particles' hydrophobicity and surface to charge ration and thus make them more reactive, compared to macro plastics. These properties not only make themselves potential toxins, but they also make them an interface or mechanical vectors for other toxic compounds persistent in aquatic environment. Several studies have investigated the toxicity of Microplastics in presence of other particles such as metals (Zhang *et al*., 2020).

Due to the strong interaction between organic pollutants and Microplastics, possible synergistic or additive effects of Microplastics on the lethal toxicities of organic pollutants have been widely studied in different organisms (Bakir et al., 2014, O'Donovan et al., 2018, D.Prokic *et al*., 2021). Various studies demonstrated that micro/nanoplastics show carrier effects that increases the concentrations of other organic or metallic pollutants taken up by organisms (Besseling *et al*., 2013; Chen *et al*., 2017; Granby *et al.,* 2018; Zhao *et al*., 2020)

and are responsible for physical damage as well as alteration of metabolic activities (Ma *et al*., 2016; Syberg *et al*., 2017). Greater histopathological damage was observed in organisms (i.e. mussels and zebrafish) exposed to pollutants (PAHs, PCBs, brominated flame retardants, perfluorinated compounds or methylmercury) together with Microplastics than to the pollutants alone (Paul-Pont *et al*., 2016; Rainieri *et al*., 2018). Moreover, owing to the different sorption/ desorption mechanism (internal partitioning/pore filling vs surface interactions), different types of Microplastics may differ in their histopathological damaging effectstogether with sorbed pollutants.

1.19.1. Toxicological Effects of Micro/Nanoplastic Pollution on Water Biota

Micro plastics take different routes to enter water bodies that include both fresh water and sea water. Currently, microplastics are ubiquitous, in streams, river mouth, on seashores, the ocean surface or in the water columns, and on the seafloor (GESAMP 2015). The pervasive nature of the micro or nano plastics in the environment makes their interaction with water biota inevitable. The bio availability of micro or nano plastics greatly depends on their size, shape and density (Wright *et al*., 2013). According to Ocean Plastics Lab 2018, over 1401 marine species are known to interact with marine plastic debris in different ways. However, entanglement and ingestion are the most common types of interaction between water biota and micro/nano plastics (Gregory, 2009). The possible effects of micro/nanoplastics on living organisms present in water include pathological stress, reproductive

complications, changes in enzymes activities, reduced growth rate, and oxidative stress (Besseling *et al*. 2014; Sutton *et al*. 2016). Relatively smaller particles (<100 nm) may pose greater consequences when being ingested, because they may end up in the tissues or even inside the cells (Lusher, 2015). Moreover, the time a particle spends inside the body (i.e., the retention time) is crucial for estimating chemical damage within the body.

Even the aquatic primary producers called phytoplankton such as algae and mosses have also been shown to interact with microplastic pollutants. Bhattacharya *et al*. (2010) have reported in their research that nanosized plastic particles can be adsorbed by a green alga (*Scenedesmus* spp.), hindering their photosynthetic activity. Microplastics can form aggregates with some phytoplankton species. The phytoplankton *Rhodomonas salina* tends to incorporate more microplastic to the aggregate compared to *Chaetoceros neogracile* (Long *et al*., 2015). More concerning effects are addressed in a recent study by Kalčíková *et al*., (2017) with a freshwater species. Sharp polyethylene microplastics from exfoliating cosmetic products are reducing the viability of the root cells of the duckweed (*Lemna minor*), which detrimentally affects their growth.

Despite this, a significant number of publications have reported detrimental effects of micro and nano plastic pollution on aquatic as well as coastline living organisms. For example, in a study, crustaceans when exposed to plastic particles were shown to have adverse effects. Their exoskeleton was

affected and with the increase in exposure time, increased mortality and reduced growth was observed. (Aljaibachi and Callaghan, 2018). To date, the major detrimental effects of micro and nanoplastics on aquatic organisms include abnormal embryonal development (Lee *et al*., 2013; Jeong *et al*., 2017), decreased lipid droplet storage (Cui *et al*., 2017), decreased feeding rates (Cole *et al*., 2015; Cole *et al*., 2013; Ogonowski *et al*., 2016; Rist *et al*., 2017), energy depletion (Cole *et al*., 2015), decreased survival (Au *et al*., 2015; Manfra *et al*., 2017), reduced growth (Aljaibachi and Callaghan, 2018; Au *et al*., 2015; Besseling *et al*., 2014; Jeong *et al*., 2016; Redondo- Hasselerharm *et al*., 2018; Ziajahromi *et al*., 2018), altered reproduction (Au *et al*., 2015; Besseling *et al*., 2014; Cole *et al*., 2015; Cui *et al*., 2017; Jeong *et al*., 2017; Jeong *et al*., 2016; Lee *et al*., 2013; Ogonowski *et al*., 2016; Ziajahromi *et al*., 2017), malformations (Besseling *et al.,* 2014), delay in molting (Jeong *et al*., 2017), abnormal swimming behavior (Rehse *et al*., 2016; Ziajahromi *et al*., 2017) and damaged intestinal microvilli (Chae *et al*., 2018).

1.19.2. Effect of Micro/Nanoplastic Pollution on Terrestrial Organisms

Like aquatic organisms, terrestrial organisms encounter micro as well as nanoplastics through different routes. Generally, the toxicological effects of micro/nanoplastics could be due to two possible reasons. (Machado *et al*., 2017). I) The release of plastic additives, plasticizers, and other components of the polymer matrix comprise one source of plastic pollutant toxicity. The

leaching of toxic components occurs during the use and degradation of macro plastics in the environment, or within organisms when being ingested or inhaled (CONTAM, 2016; Whitacre, 2014). This leaching causes serious problems because several of these chemical derivatives for example phthalates and bisphenol A are recognized for their estrogenic activity in living organisms and further potential endocrine disruption in vertebrates and some invertebrate species (Cole *et al*., 2011). Many plastic polymers have been reported to be responsible for leaching compounds that show estrogenic activity (Yang*et al*., 2011). Estrogenic and demasculinizing effects have been associated with the leached compounds in many vertebrates and invertebrates (Tracey *et al*., 2013; Marty *et al.*, 2017; Tamschick *et al*., 2016; Ziková *et al*., 2017). The wide-ranging use of plastic products and the resulting increase in the concentrations of endocrine active compounds in the environment are of great ecological concern. Endocrine systems have reasonably well preserved during the evolution of vertebrates, and therefore endocrine disruptors might trigger wide-ranging direct consequences for animal health (Kontrick, 2018). The other source of plastic pollutant toxicity is due to micro/nanoplastic particles itself.

These microplastic particles interact with the cells at molecular level i-e biological membranes, organelles, and molecules as a result several effects are incited that are usually activated by other toxins such as inflammation, changes in membrane permeability,oxidative stress ((Forte *et al*., 2016; Jeong

et al., 2016). Furthermore, the internalization of nanoplastics might increase the cytotoxicity by allowing direct interaction of nano plastic particles with genetic material. (Syberg *et al.,* 2015). Certainly, the micro/nano plastic particle exposure in various human and non-human models have been reported to show changes in expression of genes, inflammatory and biochemical responses, as well as carcinogenesis (Forte *et al*., 2016).

1.19.3. Micro/Nanoplastic Pollution with Respect to Human Health

Human exposure to micro/nanoplastic pollution has become inevitable. The plastic pollutants present in multiple compartments of environment take different routes to enter human body i-e ingestion of micro/nano particle contaminated food, inhaling the micro/nano particles present in air or through skin (Revel *et al*., 2018).

Among the three routes for plastic particle entry, the major one is through ingestion of contaminated food (Galloway, 2015). The ingested particles reach the gastrointestinal track where it triggers inflammatory response, increases membrane permeability, and possibly alters gut microbiota (Salim *et al*., 2013). According to an investigation by Forte *et al*., 2016 on gastrointestinal adenocarcinoma cells, plastic particles when ingested leads to change in gene expression, rendered cell viability as well as activation of inflammatory response. To date, a number of food items have been reported to contain plastic particles such as mussels (Li *et al*., 2016), commercial fish (Neves *et al*., 2015), as well as table salt (Karami *et al*., 2017),

sugar (Liebezeit and Liebezeit,, 2013), and bottled water (Oßmann *et al*., 2018), since these items are basic food necessities so their ingestion is inevitable. Inhalation of micro and nano plastic particles through air is also an important route of entry. Air has been contaminated with plastic pollutants through different sources such as synthetic textiles, abrasion of car tires, buildings, and resuspension of microplastics in surfaces. After being inhaled, the plastic particles are carried to respiratory track where their deposition occurs (Prata *et al*., 2020). The deposition of plastic micro particles depends upon the particle properties such as size, surface area and density where the less dense and smaller particles found to be carried deeper in lungs (Prata *et al*., 2020). Particles after being deposited could undergo clearance by macrophages or enter the circulatory and lymphatic system and hence translocated to other body parts (Prata *et al*., 2020). Thus, under higher concentration, the inhaled plastic particles can cause lesions in respiratory system (Prata *et al*., 2020). The dermal route of entry for microplastics has been reported as less significant, although it has been speculated that nanoplastics (<100 nm) could transverse the dermal barrier (Revel *etal*., 2018).

Microplastic internalization into cell trigger antioxidant response and thus leads to oxidative stress. The reason for this oxidative stress could be the large surface of internalized plastic particle, release of adsorbed oxidizing species such as heavy metals, or production of ROS in response to the inflammatory

response activated by ingested plastic particle (Kelly and Fussel, 2012; Valavanidis *et al*., 2013). The oxidative stress results in cytotoxicity.

1.20. Aims and Objectives:

Based on the literature, the present research aims to qualitatively assess freshwater samples from River Mersey, for particles. The study focused on detection and identification of microplastic particles in river water samples. Subsequently, the study evaluated the impact of identified microplastic particles and their combination with heavy metals on freshwater invertebrate *Daphnia pulex*.

Given that, this study has the following defined objectives.

1. Detection and identification of microplastics in surface water samples (River Mersey) using established methodologies (light microscopy, fluorescence microscopy, micro-Raman spectroscopy).

2. To develop a basic bioassay protocol for animals exposed to environmentally realistic amounts of microplastics on selected model invertebrate (*Daphnia pulex*) (including developmental parameters, survival, reproduction, and heartrate).
3. To perform genome-wide transcriptome analysis using RNA-illumina sequencing based RNA seq, to evaluate the effects of microplastic exposure at precisely defined concentration, on range of molecular pathways (which includes oxidative stress, development, and reproduction).

4. To evaluate the effect of microwave assisted thermal treatment on degradation of microplastics and the toxicological impact of degraded microplastics on *Daphnia pulex*.

CHAPTER 2

DETECTION AND IDENTIFICATION OF MICROPLASTICS INFRESHWATER SAMPLE

2.1. Introduction

2.1.1. Methods for Characterization and Quantification of Microplastics in Water Samples

Quest for characterization and quantification of micro/nanoplastics in complex environmental samples such as soil, sediment, fresh water, organs, and tissues is thriving recently. Lack of effective techniques to deal with the sample complexity limits the study of micro/nano plastics. There are few techniques in use for identification and quantification of M/NPs that includes visual sorting/identification using microscope, Fourier transform infrared spectroscopy (FTIR), Raman spectroscopy (Xu *et al*., 2019) and Mass spectrometry (Hendrickson *et al*., 2018), Nanomagnetic Resonance (NMR) (N. Peez and W. Imhof, 2020).

2.1.2. Visual Sorting/Identification

Visual sorting or identification is the conventional technique that has been in use for initial screening of micro plastic particles in environmental samples. The visual scanning is done under stereo microscope after the samples are being processed by density separation technique. The visual sorting technique has limitation as it could detect relatively larger micro plastic particles that range in size 1-5 mm with almost 70% chances of error (Hidalgo-Ruz *et al*., 2012). Moreover, the sorting efficiency decreases with decrease in particle size (Hidalgo-Ruz *et al*., 2012). Thus, it doesn't remain the only choice for efficient

characterization or quantification of micro or nano plastics in water samples.

2.1.3. Fourier Transform Infrared Spectroscopy (FTIR)

One of the advanced techniques used for characterization and quantification of micro plastics in environment samples such as water is Fourier transform infrared spectroscopy (FTIR) (Tagg *et al*., 2015). With the help of latest advancements in micro-Fourier transform infrared (μ-FTIR) imaging technique, the automatic detection of micro plastic particles has become possible without presorting (Vianello *et al*., 2019; primpke *et al*., 2017). Moreover, a significant decrease in analysis time and increased accuracy of the results is possible nowwith the focal plane array–based μ-FTIR imaging of whole filter surfaces (Primpke *et al*., 2017).

For larger micro plastic particles, conventional FTIR technique is used whereas for smaller particles micro-FTIR technique is required (Okoffo *et al.,* 2019). Currently three modes of FTIR techniques are in use for micro plastic particle analysis i-e transmittance (Kappler *et al.*, 2015), reflectance and attenuated total reflection (ATR) (Kappler *et al*., 2018). In transmittance mode, the particle under investigation needs to be sufficiently thin such as less than 100 μm to avoid total absorbance in FTIR spectrum. In contrast with this, the reflectance and attenuated total reflectance ATR mode does not the particle under investigation to be sufficiently thin (Shim and Hong, 2017). For particles with a relatively planar surface the reflectance mode is more suitable whereas those with irregular surfaces ATR is needed (Harrison *et al*., 2012).

FTIR spectroscopy allows the study of molecular composition of microplastic samples by using infrared radiation. Dry sample 5 to 10 mg is placed on the holder to allow IR pass through the sample. The molecules of plastic polymers absorb infrared radiation in a specific manner thus producing infrared spectrum (Veerasingam *et al.*, 2020). An infrared spectrum represents a fingerprint of a microplastic sample with absorption peaks corresponding to the frequencies of vibration between the bonds of the atoms making up the material. Because each different polymer material is a unique combination of atoms, no two compounds produce exactly the same infrared spectrum. Therefore, the chemical structure of a polymer molecule can be determined by FTIR (Chalmers, 2006). The pretreatment of microplastic samples is done to reduce spectral interference by removing coenriched organic or inorganic particles from microplastic surface (He *et al.*, 2018). For this purpose, different regents have been compared previously, such as ethanol (Corami *et al.*, 2020) 30% H_2O_2 (Tagg *et al.*, 2015) 65% HNO_3 (Scheurer and Bigalke, 2018) Fenton's reagent (Hurley *et al.*, 2018)], and enzymatic digestions (Von *et al.*, 2013).

2.1.4. Mass Spectrometry (MS)

Although mass spectrometry (an analytical technique that is used to measure the mass-to- charge ratio of ions) is applicable in detection and quantification of micro/ nano plastic particles - conventional mass spectrometry is often not a good choice. The plastic polymers are high in

density with high molecular weight; thus, the detection is not possible using the conventional MS technique (Duemichen *et al*., 2018). Rather there are various advanced types of mass spectrometry that work either themselves or in combination with other advanced techniques for efficient quantification of plastic particles such as Pyrolysis-GC–MS. In this method, the decomposition of the sample is carried out in a small furnace or tube wrapped by a resistance wire for heat transfer. The gaseous decomposition products are transferred through a heated transfer capillary into the GC–MS system (Fabbri *et al*; 2000). There are a handful number of methods that are still under experimental trials to be described as efficient for detection and quantification of plastic particles. The present research also intends to devise such quantification techniques.

2.1.5. Differential Centrifugal Sedimentation (DCS)

Differential centrifugation sedimentation is an advanced technique that measures size distribution of nanoparticles in a given sample with very high resolution. It measures the time it takes for the particles to sediment through density gradient where the length of time depends on the size and density of nanoparticle. With recent advances in DCS technology, it allows analysis of nanoparticles down to 2 nm with size difference as little as 2 %.

The general principle of DCS is that the sample is added to a hollow, optically clear disc which contains the compatible density gradient and is driven by variable speed motor. When the sample accelerates towards the density

gradient, it spreads. As the sample reaches the fluid gradient surface, sedimentation of individual particles started taking place. The length of sedimentation path gives the size.

of nanoparticle, if the sedimentation path is longer, then the light beam is expectedly seeing a smaller 'segment' (more correctly, a smaller differential) of the whole distribution. The path length of sedimentation is automatically calculated based on the sedimentation time of the calibration standard and the viscosity of the liquid.

Although this method has been of great use for studying different nanoparticles (metallic, biological or polymer based) (Kestens *et al*., 2017), its use for analysis of nanoplastics in environmental samples has not been reported yet.

2.1.6. Bias in Micro/Nanoplastics Detection Methods

Although, a number of techniques are currently in use for detection, characterization and quantification of micro and nano scale plastic particles in environmental samples, there arelimitations with these techniques and thus extensive research is requires to deal with these limitations to give more accurate results.

For instance, the FTIR technique, it is quite advanced and has been of great help in investigating micro plastics but the limitation with this technique is that it is very laborious and time-consuming for whole-sample particles detection

and with decrease in size of particle under investigation, the detection time increases. Moreover, the complexity of media might interfere with the results accuracy such as the spectral peaks obtained from a sample that contain coenriched particles (organic debris or minerals) might be dominated by non-plastic particles and thus might give false negative results (Duemichen *et al.*, 2019). GC-MS serves as a more powerful and advanced technique for investigation of micro as well as nanoparticles but like other techniques they also have some limitations that restrict its ability. As discussed above Pyrolysis GC-MS gives promising results in providing molecular information of indicative fragment ions to analyze different types of plastics but it cannot process more complex samples (Fisher and Scholz-Böttcher, 2017). Moreover, it results in sample destruction and sample is lost after analysis. Thermal desorption GC-MS combine with thermogravimetry serves better with more complex samples (Dümichen *et al.*, 2015). For river water samples, the samples need to be concentrated enough to be detected by the instrument as the lower detection limit for micro/nanoplastics in environmental samples has not been measured. If there is very low micro/nano plastic concentration in a sample, it would be hard to detect and thus may give false negative results.

A high throughput research is required for devising novel strategies that can meet the advance requirements for micro and nano plastic particles investigation.

This section of study aims to validate the use of established analytical techniques for microplastic detection and identification in water samples. This

section also examined the water samples for presence of heavy metals. Heavy metals will be used as other potential pollutants expected to be transported to other organisms by binding or adsorbing to the surface of microplastics.

2.2. Materials and Methods

2.2.1. Sample Collection

The sample collection was done from River Mersey. River Mersey is situated in the Northwest of England, stretching from Stockport, Greater Manchester and ends at Liverpool Bay, Merseyside. The river flows through sub-urban areas of Manchester, covering an area of approximately 4500 km^2. The river enters the Irish Sea after a total course of 70 miles (110 km). The presence of several WWTPs near the river Mersey makes it suitable to assess its role in fate and transport of Microplastics to other trophic levels.

Five sites, starting from top of river to downwards were selected with approximate distance of about 15 kms. Selected sites include Ashton Upon Mersey, Otterspool, Spike Island, Fiddlers ferryand Pickering pasture.

The sampling method described by Scherer et al., 2020, was used with some modifications. Plankton net (stainless steel frame diameter of 300mm and an 880mm long bag,) with mesh size of 53 µm was purchased from NHBS wildlife/ecology/conservation and used for collection of water samples. The plankton net was placed right below the water surface for 10 minutes. The river surface was sampled over approximately 10 m.

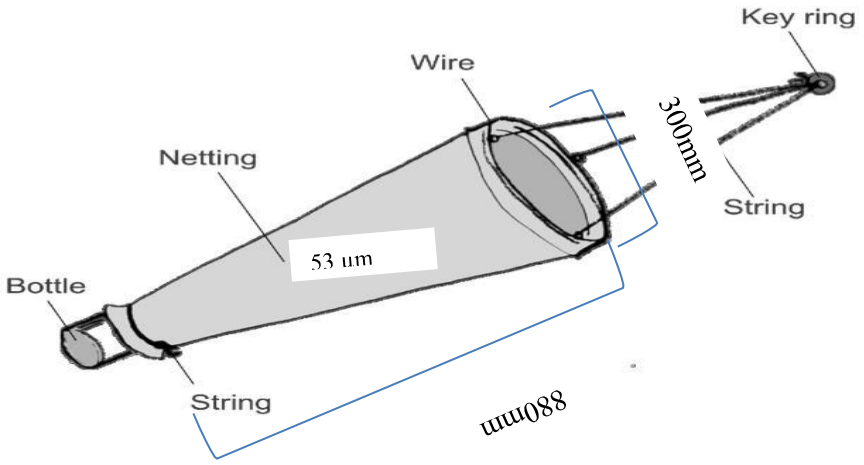


Figure 2.1: Figure of plankton net used for collection of freshwater samples.

The filtered water volume was calculated using following formula;

Volume of water filtered = Water velocity (m/s) × opening of the net [m^2] × 1000.

0.166 (m/s) × 0.6 (m^2) × 1000 = 99.6 litres/sec (~0.1 m^3)

The content captured on the net was poured in a steel bucket and rinsed thoroughly with the same riverwater. Any bigger objects such as stones, sticks or leaves were removed. The rest of the sample water was strained through stainless steel strainer with mesh size of 5mm and stored in glass jars with metallic lids. Three samples from each site were collected. Samples were kept in refrigerator at 4^{O}C until processed. The stored water samples were termed as "concentrated water sample" (since the small volume of water obtained through above sampling method depicts larger water volume under investigation) in subsequent analysis.

2.2.2. Sample Preparation

Fresh water samples are often rich in natural particles that might include undigested biogenic organic matter (mostly vegetal litter such as algae, leaves, propagules, driftwood,(Kristensen et al., 2008, Duan et al., 2020) and mineral material, which can clog filters, float with density solutions (Lagarde et al., 2016), trap microplastics in biogenic organic matter aggregates, and cause mis identification of microplastics (Li et al., 2018). Thus, it is very important to devoid the samples of these natural particles. The following procedure was adapted to process the samples.

2.2.2.1. Organic matter digestion

The method used for digestion of organic matter was as described by *Stanton et al., 2019*. According to the method, 200 ml of concentrated freshwater sample was treated with 50 mL of 30% H_2O_2 (Sigma-Aldrich) and was gradually heated to 75 °C for 4−5 h. The method was validated in an investigation by Hurley et al, to access efficacy of different reagents (H_2O_2, Fenton's reagent and KOH) for organic matter removal alongside their effect on polymer particles. (Hurley *et al.,* 2018). After being left to cool overnight, these samples were decanted in clean glass beakers.

2.2.2.2. Density Separation

As microplastics are low density polymers (0.8–1.4 g/cm³), they tend to float in denser salt solutions and hence can be separated using relatively denser

salts. In this study, $ZnCl_2$ (Sigma- Aldrich, density 1.4-1.6 g/cm³ w/v) was used for the separation (*Maes et al., 2017*). The salt solution was mixed with sample in glass separating funnel in 2:1 and left to settle overnight. The top layer was extracted and filtered onto 0.45 µm mixed cellulose ester gridded filter papers (Whatman ME 25/41) using a glass vacuum filtration apparatus.

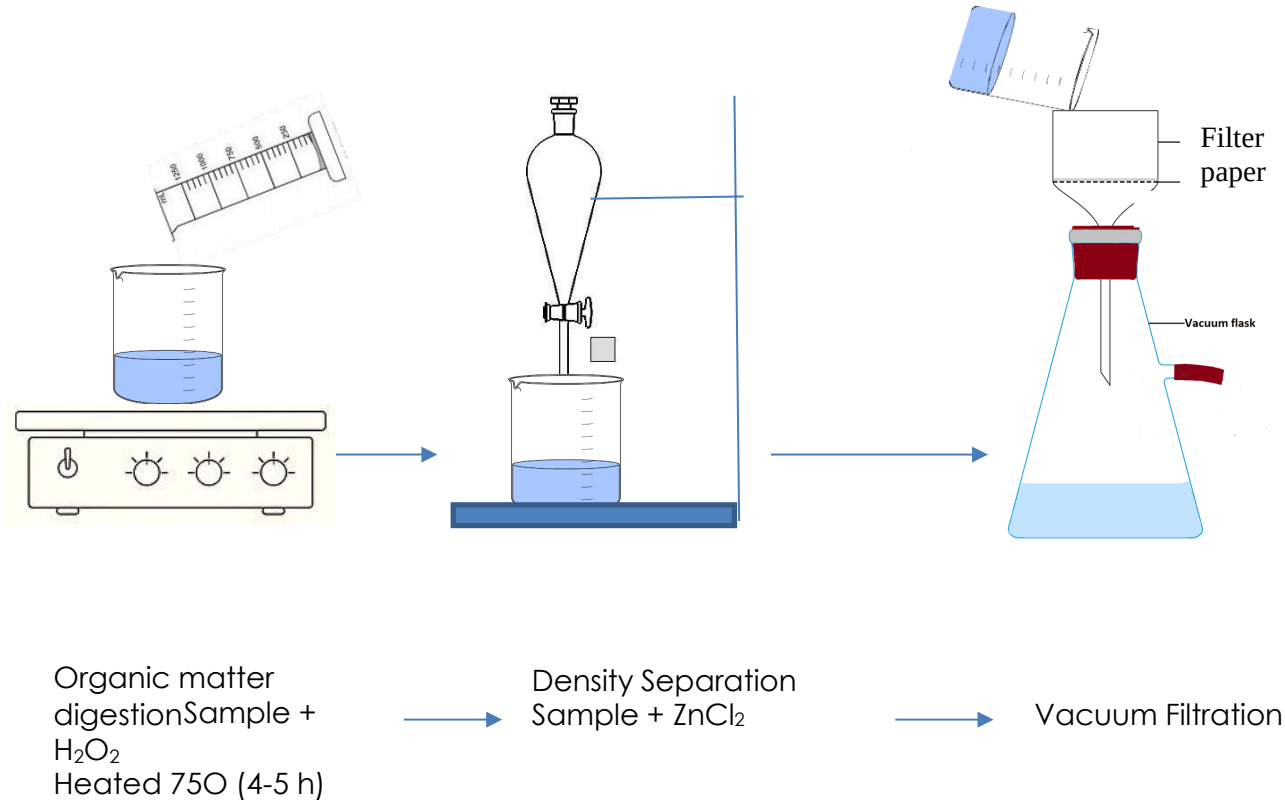


Figure2.2: Method for fresh water sample processing in laboratory

2.2.3. Elemental Analysis

River water is a very complex environmental sample because of the presence of varying concentrations of anions, cations or heavy metals and many other pollutants. Lin et al., 2021 showed that microplastics have a pH point of zero charge (pH pzc) around 3, which implies that in the natural aqueous environment, microplastics carry a negative charge on their surface (Lin et al., 2021). Hence their interaction with cations in the natural environment is most likely. However, under different pH conditions, the surface charge of Microplastics could vary. For example, as stated by Liu et al., 2021, when the pH is less than 3, the surface of the microplastics (PE) is positively charged, and the zeta potential and electrostatic repulsion are low. In such a scenario, they might interact with anions. Hence, a complete elemental analysis was performed for river water samples to detect presence ofcations and heavy metals.

For heavy metals, Inductively Coupled Plasma- Optical Emission Spectroscopy (ICP-OES) analysis was performed. Multi-element analysis of metals in sample solution was achieved using ICP spectroscopic technique. The basic principle of the technique is to measure element-specific light emitted by the metals in the sample.

To prepare the samples, 75 ml of each sample water was filtered with syringe filter (0.45 um pore size) and mixed with concentrated HNO_3 (69%, purchased from APC supplier) to achieve total volume of 100 ml. Commercially

available calibration standards for Ag, Al, As, Ba, Be, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Ni, Pb, Se, Sb, Sr, Ti, Tl, V and Zn (Sigma Aldrich) were used for analysis in five concentrations 0.05, 0.1, 0.5, 1.0 and 2.0 ppm . The prepared sample solutions were nebulized, and the resultant aerosol was transported to the plasma torch where the spectrum of analyte specific atomic-lineemissions was produced (Figure 2.3). The spectrum is dispersed by a diffraction grating and the intensities of the individual emission lines are monitored by photomultiplier tubes. Results are recorded as ppm for each sample solution.

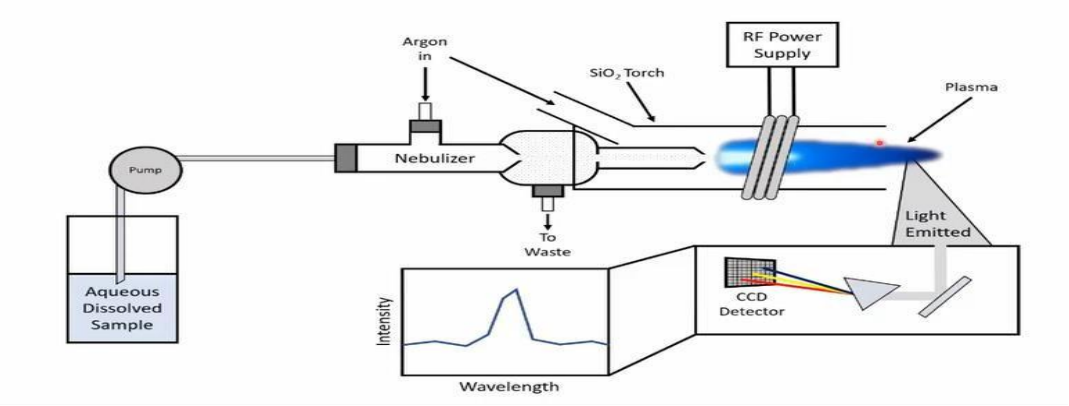


Figure 2.3: Schematic representation of ICP-OES technique performed for metal analysis in freshwater sample.

Cation's analysis was performed for detection of Li+, Na+, NH4+, K+, Ca+ and Mg+.

2.2.4. Detection and Identification of Micro/Nanoplastics in Freshwater Samples

For detection and identification of microplastics in river water samples, established methodologies were adapted.

2.2.4.1. Light Microscopy

After filtration, the first analysis performed in search of micro/nanoplastics was light microscopy. A dissecting microscope with an attached camera was used for analysis. Before analyzing the samples under microscope, it was made sure that all filter papers containing samples are dried. Inspection was done at 40X magnification. The criteria used for selecting particles as plastics was as described by *Hidalgo-Ruz et al., 2012*, which are listed below,

1. Size <5mm
2. No cellular structure visible.
3. Fibers show uniform thickness throughout the length.
4. Fibers exhibit homogeneous color throughout the length.
5. Sharp clear object boundaries.

2.2.4.2. Nile Red Dye staining

One replicate of each sample was used for staining with NR dye to be observed under fluorescence microscope. For staining, the 5ml of the sample was mixed in 50 µl of NR dye solution in acetone (Sigma Aldrich) and incubated on a Heidolph Rotamix shaker at 100 rpm for 60 minutes. Samples were then vacuum filtered (Whatman 47mm cellulose filter membrane 0.45µm).

The stained samples were viewed under a blue light (Crime Lite: 450–510nm)

through an orange filter (529nm) and expected microplastics were counted (*Maes et al., 2017*). The filters were also photographed. As a control, samples without stain were checked on same wavelength for autofluorescence.

2.2.4.3. Micro-Raman (μRaman) Spectroscopy

For chemical identification of the particles observed in light microscopy and NR staining, μRaman spectroscopic analysis was done. Thermo Scientific DXR Raman Microscope (laser wavelength 532nm) was used for the analysis. The filter papers were analysed under microscope attached to Raman Spectroscope at 50X magnification. The suspected particles were used to generate raman spectra. A library search was performed on all of the spectra collected from sample filter papers. The commercial reference libraries used for the search are listed below:

- Aldrich Condensed Phase Sample Library
- Aldrich Vapor Phase Sample Library
- Georgia State Crime Lab Sample Library
- Hummel Polymer Sample Library
- Organics by RAMAN Sample Library
- Raman Sample Library
- Renishaw Forensic Materials Database
- Renishaw Minerals and Inorganic Material Database
- Renishaw Polymeric Materials Database

- Sigma Biological Sample Library

2.3. Results

2.3.1. Elemental Analysis

All samples contained low concentrations for detected metals. Potassium (K) and Magnesium (Mg) were present in all five samples with concentration 161.15 ppm as highest found in Spike Island and 4.07 ppm as lowest in Aston upon Mersey sample. A relatively high concentration for Mg (71.09) was also observed in Spike Island sample where Ashton upon Mersey having lowest concentration given as 4.98 ppm. This could be due to the location of Spike Island on top of Mersey estuary and Ashton Upon Mersey being the farthest down the estuary. Ashton upon Mersey was the only sample that showed presence of Fe and Mn with concentration 15.53 and 3.59 ppm respectively. There is no recent data on heavy metal contamination levels of river Mersey and hence these results provided evidence for presence of heavy metal contamination.

Table 2.1: Concentration of heavy metals (Ag, Al, As, Ba, Be, Cd and Co) in river water samples. Standard 1, 2, 3, 4, 5 are commercial standards for heavy metal analysis; distilled water used as control

Sample	Ag (PPM)	Al (PPM)	As (PPM)	Ba (PPM)	Be (PPM)	Cd (PPM)	Co (PPM)
Blank	0	0	0	0	0	0	0
Standard 1	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Standard 2	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Standard 3	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Standard 4	1	1	1	1	1	1	1
Standard 5	2	2	2	2	2	2	2
Ashton Upon Mersey	0.09	0.04	0.01	0.02	-0.01	0	0
Pickering pastures	0.06	0.03	0.01	0	-0.01	0	0
Spike Island	0.09	0.02	0.02	0.01	-0.01	0	0
Fiddlers ferry	0.08	0.04	0.01	0.02	-0.01	0	0
Otters Pool	0.06	0.23	0.01	0.01	-0.01	0	0

Table 2.2: Concentration of heavy metals (Cr, Cu, Fe, K, Mg, Mn, Mo and Ni) in river water samples. Standard 1, 2, 3, 4, 5 are commercial standards for heavy metal analysis; distilled water used as control

Sample	Cr (PPM)	Cu (PPM)	Fe (PPM)	K (PPM)	Mg (PPM)	Mn (PPM)	Mo (PPM)	Ni (PPM)
Blank	0	0	0	0	0	0	0	0
Standard 1	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Standard 2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Standard 3	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Standard 4	1	1	1	1	1	1	1	1
Stndard 5	2	2	2	2	2	2	2	2
Ashton Upon Mersey	0	0	15.53	4.07	4.98	3.59	0	0
Pickering pastures	0	0.01	0.02	5.56	9.52	0	0	0
Spike Island	0	0.01	0	161.15	71.09	0	0	0
Fiddlers ferry	0	0.01	0.01	8.59	15	0	0	0
Otters Pool	0	0.07	0.46	53.57	54.23	0.04	0	0

Table 2.3: Concentration of heavy metals (Pb, Se, Sr, Ti, Tl, V and Zn) in river water samples. Standard 1, 2,3, 4, 5 are commercial standards for heavy metal analysis; distilled water used as control

Sample	Pb (PPM)	b (PPM)	Se (PPM)	Sr (PPM)	Ti (PPM)	Tl (PPM)	V (PPM)	Zn (PPM)
Blank	0	0	0	0	0	0	0	0
Standard 1	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Standard 2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Standard 3	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Standard 4	1	1	1	1	1	1	1	1
Standard 5	2	2	2	2	2	2	2	2
Ashton Upon Mersey	-0.01	0	0	0.09	0	0.01	0	0.01
Pickering Pastures	-0.01	0	0.01	0.14	0	0.01	0	0.03
Spike Island	-0.01	0	0.02	2.08	0	0.02	0.01	0.02
Fiddlers ferry	-0.01	0	0.01	0.26	0	0	0	0.02
Otters Pool	-0.01	0	0	0.71	0.01	0.01	0	0.05

Table 2.4: Concentration of Cations (Li+, Na+, NH4+, K+, Ca+ and Mg+) in river water samples. Standard1, 2, 3, 4, 5 are commercial standards for heavy metal analysis; distilled water used as control

Sample	Lithium (ppm)	Sodium (ppm)	Ammonium(ppm)	Potassium(ppm)	Calcium (ppm)	Magnesium(ppm)
Standard	5.021	19.979	25.152	25.194	6.044	50.028
Blank	0.022	0.095	0.037	0	0.194	0
Ashton Upon Mersey	0.034	39.978	31.587	1.717	0	36.379
Pickering pastures	0.026	192.064	0	27.923	0	63.015
Spike Island	0	0	0	0	0	87.304
Fiddlers ferry	0.020	214.213	0.028	0.068	0	37.134
Otters Pool	0	0	1453.819	0	0	80.992

2.3.2. Light Microscopy

All the samples were detected positive for microplastics according to the criteria mentioned above. Among all five sites, Ashton samples showed the highest number of microplastic particles i-e 290 particles/m^3. Whereas lowest number of microplastics was observed in Spike Island and Otterspool samples given as 45 and 35 particles/m^3 respectively. Following order for the distribution of different types of microplastics was observed in samples under investigation; Ashton Upon Mersey > FiddlersFerry > Pickering Pasture > Spikes Island > Otters pool.

Table 2.5: Microplastic count, type and approximate size in river water sample (five selected sites)

Sample (200ml)	Microplastics count (n)							~ size (um)		
		Pellets/beads	Fibres	Fragments	Other	Total (/0.1 m^3)	Particles/ m^3	<100	100-200	>200
	Blank	0	3	1	0	3		1	0	0
Ashton Upon Mersey	Rep1	2	30	0	0	32	290	30	2	0
	Rep2	1	23	2	0	26		23	3	0
Fiddlers Ferry	Rep1	5	6	0	1	12	140	11	1	0
	Rep2	4	12	0	0	16		16	0	0
Spikes Island	Rep1	0	3	1	0	4	45	1	3	0
	Rep2	1	3	1	0	5		4	1	0
Otters pool	Rep1	2	4	0	0	6	35	5	1	0
	Rep2	1	0	0	0	1		0	1	0
Pickering Pasture	Rep1	0	7	0	0	7	100	6	1	0
	Rep2	0	13	0	0	13		13	0	0

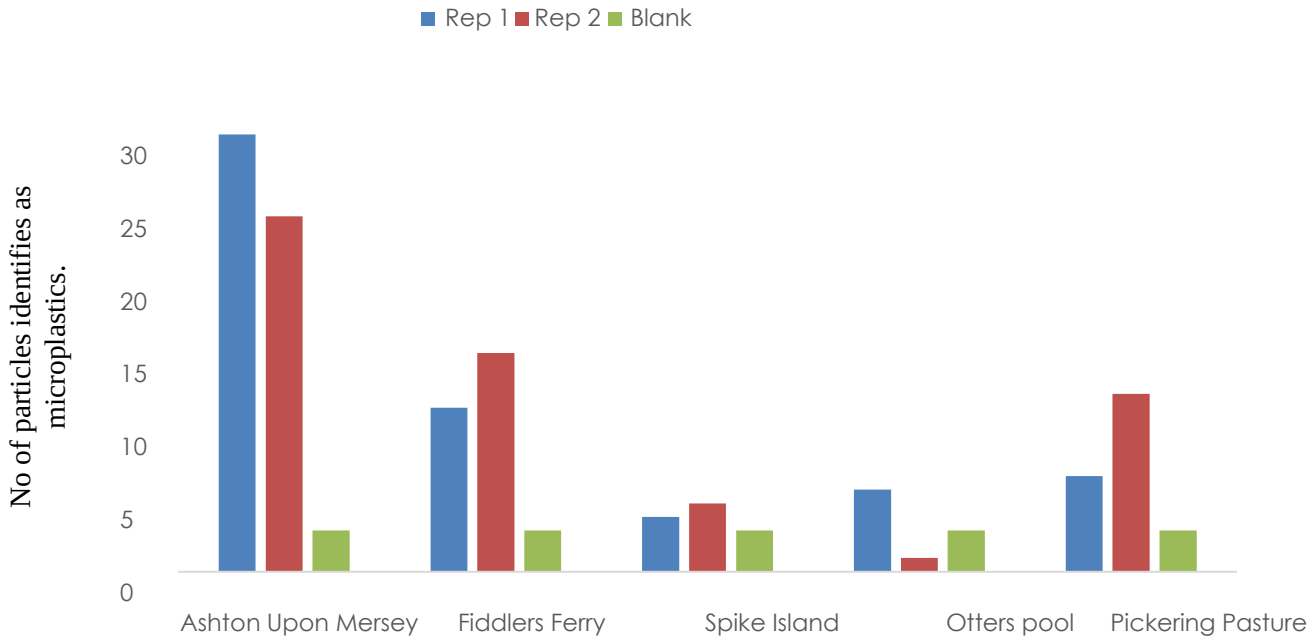


Figure 2.4: Graphical Representation of concentration of particles observed in five samples; y-axis representsnumber of particles.

Based on their shapes, observed microplastics were classified as fragments, fibers, Pellets and those which were difficult to classify were classed as others. This classification could be of importance as it might indicate the parent materials of the microplastics found in samples. (Zhang et al., 2018).

Fibers were most abundant in all studied samples (Figure 3.2). However, very few particles of other shapes such as fragments and pellets were observed. Moreover, only light microscopy could not be considered as reliable method for these beads/pellets particle identification because these particles could be mistaken with non-plastic, metallic particles as they exhibit similar physical properties (Blair et al., 2019). Determination of size with this visual technique was very difficult because the particles exhibited round or bended appearance. Hence a lengthwise approximation was made using a scale

bar in µm unit. The scale bar was obtained from image analysis software of fluorescence microscope at same magnification (40X) and duplicated to each image in *imageJ* software. For convenience, three size categories were made; <100 µm, 100-200 µm and >200 µm.

Most of the particles were less than 100 µm, a very few particles were between 100 to 200 µm whereas no particles larger than 200 um were seen in any samples (table 3.5).

Figure 2.4 presents a visual depiction of the concentration of particles detected in five samples, with the y-axis indicating the quantity of particles. Figure 2.5 illustrates the relative concentration of types of plastic based on their shape.

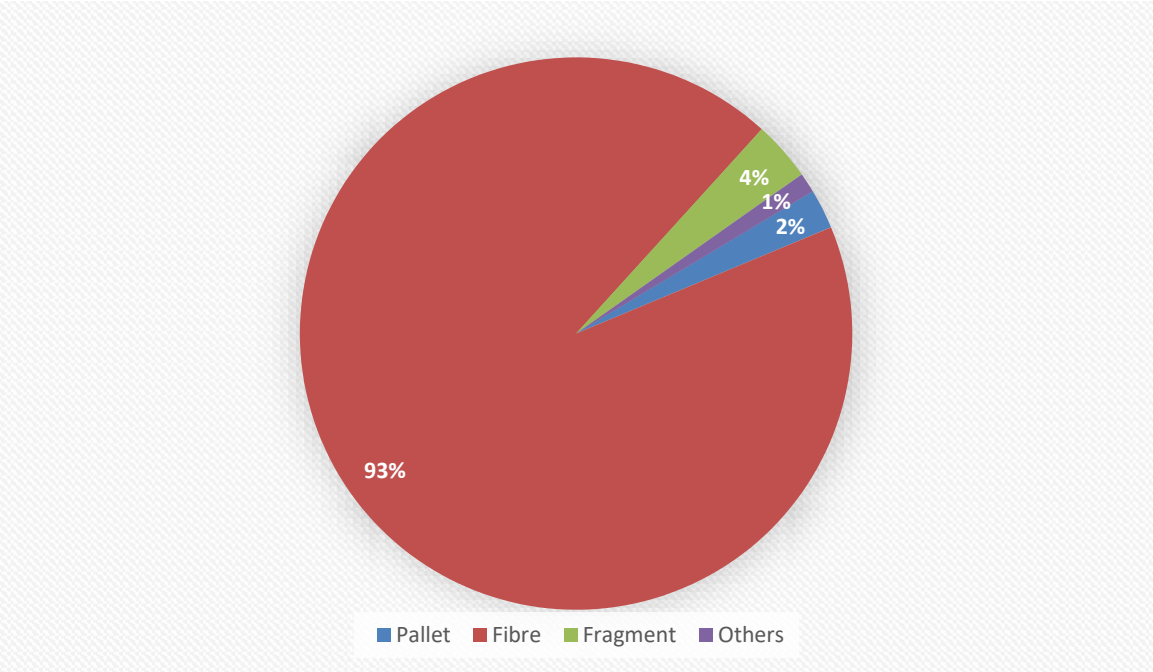


Figure 2.5: Pie chart representing the percentage of each suspected plastic type observed among all riverwater samples.

The following images were captured through the camera attached with the microscope used for visual analysis at 40X magnification. Scale bar was added in µm unit to give estimate of the observed particlesize.

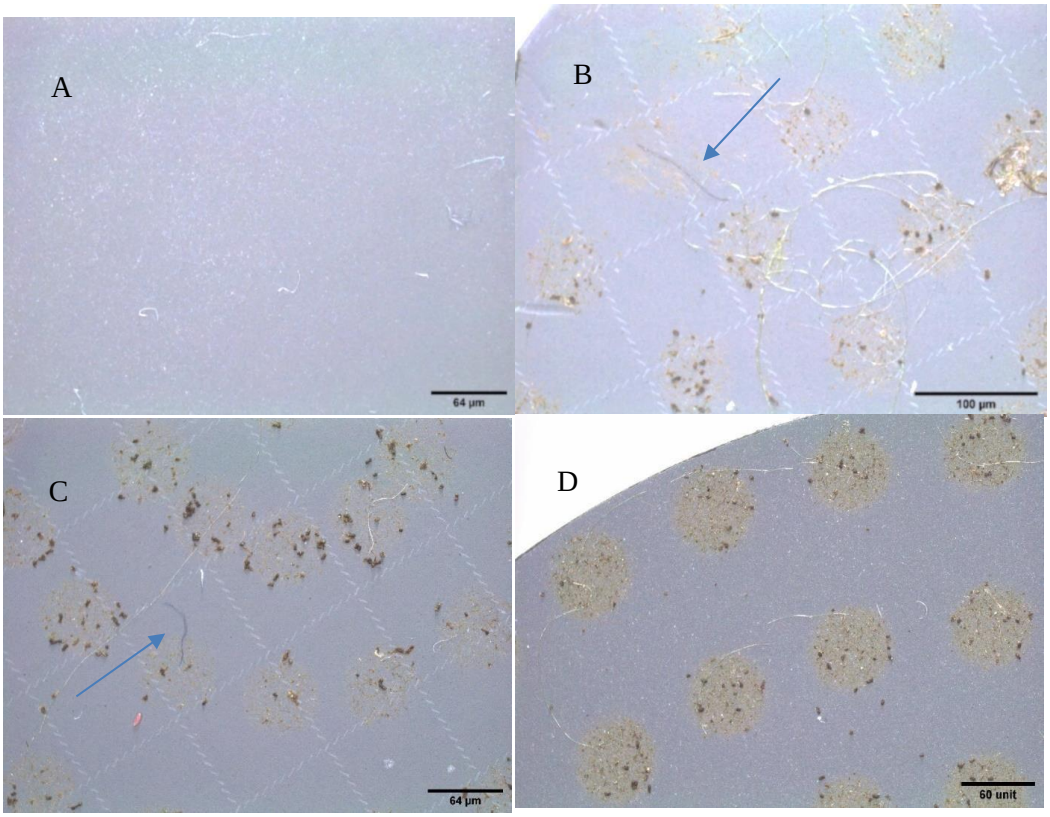


Figure 2.6: Filter paper images of Ashton Upon Mersey sample at 40X magnificationunder dissecting microscope; In section A and B, blue arrow points to a blue microplastic fiber,

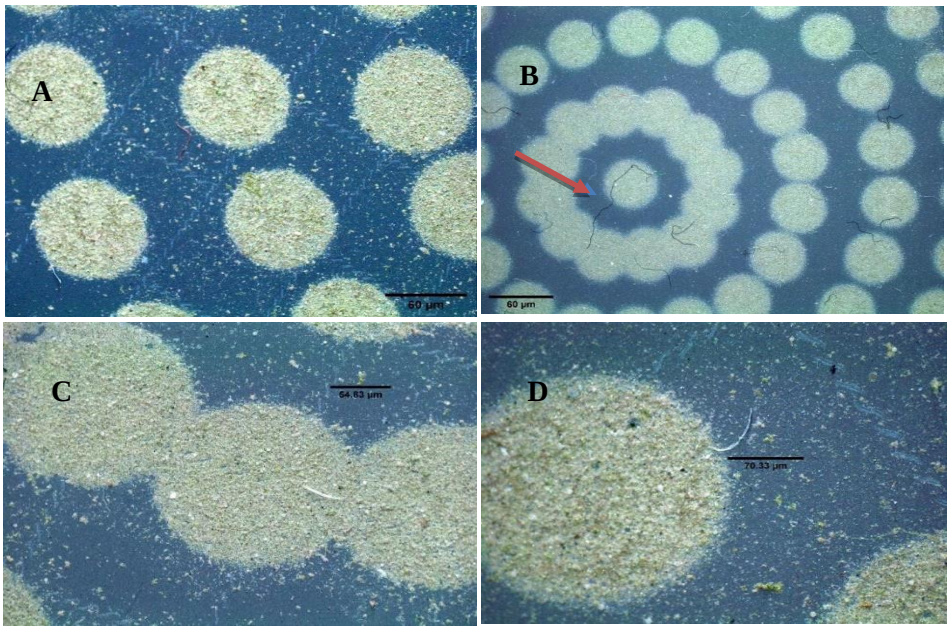


Figure 2.7: Filter paper images of fiddlers Ferry sample at 40X magnification under dissecting microscope, in section B, red arrow points to a suspected microplastic fiber

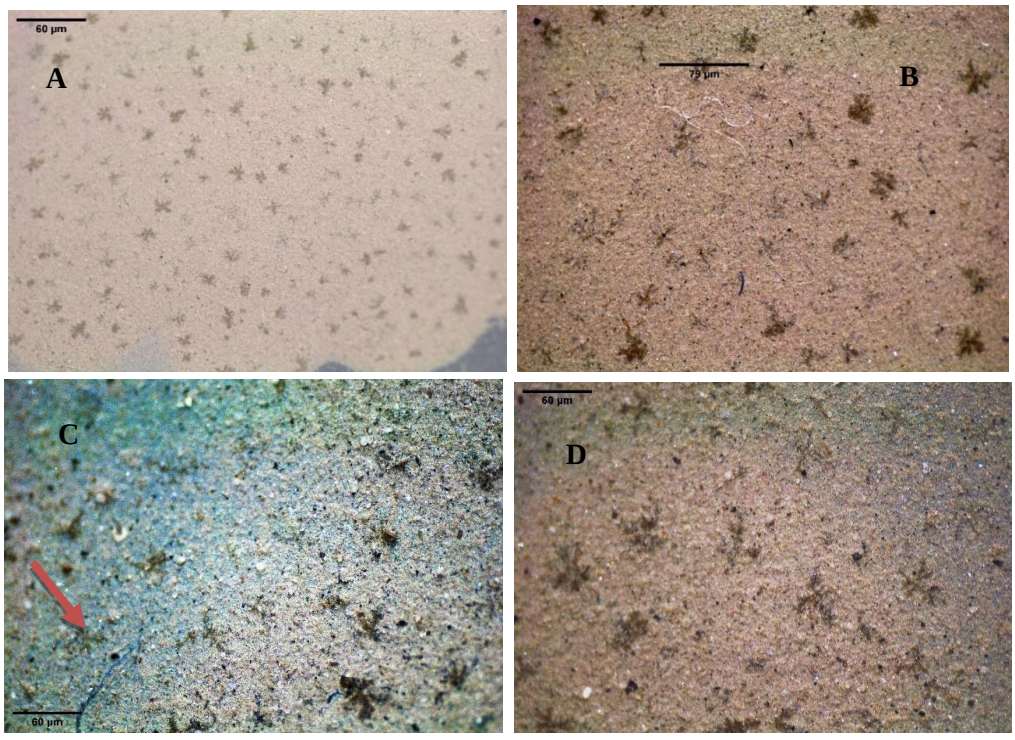


Figure 2.8: Filter paper images of Otters pool sample at 40X magnification under dissecting microscope, In section C, red arrow points to a bright blue fiber suspectedas microplastic

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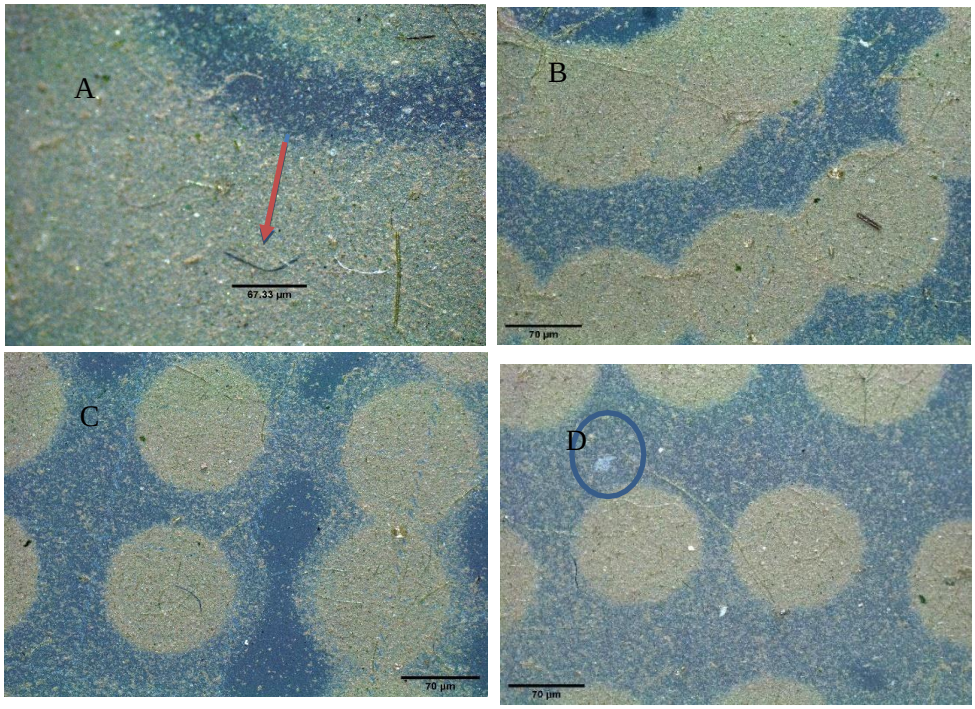

Figure 2.9: Filter paper images of Spike Island sample at 40X magnification under dissecting microscope, Section A, red arrow points to a dark blue fiber, suspected asmicroplastic; Section D, blue circle represents a suspected microplastic fragment

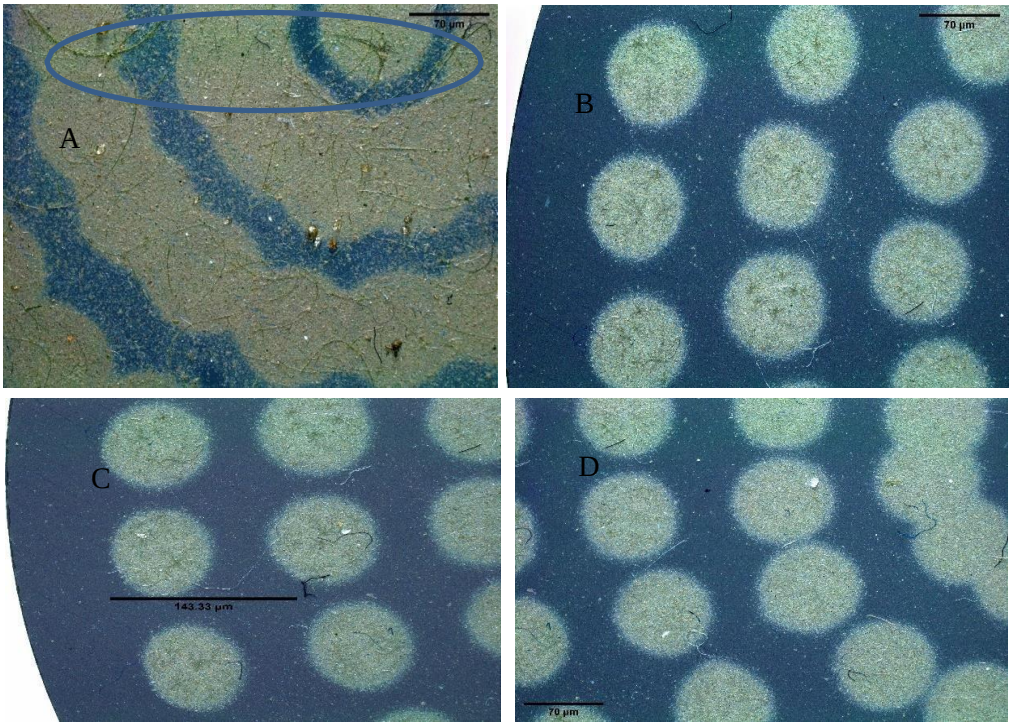

Figure 2.10: Filter paper images of Pickering pasture samples at 40 X magnification,section A, blue circle shows cellular debris

Overall, the Microplastics concentrations observed in this study was relatively low as compared to other studies (Desforges et al., 2014; Zhao et al., 2014; Tsang et al., 2017; Simon-Sánchez et al., 2019; Egessa et al., 2020; Pan et al., 2020; Suresh et al., 2020).

2.3.3. NR Staining

All the particles that were suspected to be microplastics under light microscopy were observed to be stained with dye. The microplastics count in all the samples was high under fluorescence microscopy (table 2.6) as compared to light microscopy, particularly for one site (otters pool) the results were significantly contrary to light microscopy findings. This could be due to staining of organic particles present in the sample. Some studies suggest that (Maes *et al*., 2017) nile red has been shown to stain natural, lipid containing particles in environmental samples (Erni-Cassola et al., 2017; Burson et al., 2018). Even after sample pretreatment with H_2O_2, material stained by NR was abundant in river water samples. Because H_2O_2 naturally occurs in aquatic environments, eukaryotic phytoplankton such as dinoflagellates produce peroxidase enzymes to counteract its damaging effects (Matthijs *et al*. 2012) which include cell lysis (Burson *et al*., 2018). They are therefore resistant to H_2O_2. However, their presence in samples stained with Nile red could give an overestimation of suspected particle abundance (Shim *et al*., 2019) which was observed in all samples. There was no evidence found in literature about Nile red dye staining metals, hence it could not be stated that the

overestimation observed in this result includes the metals staining or not.

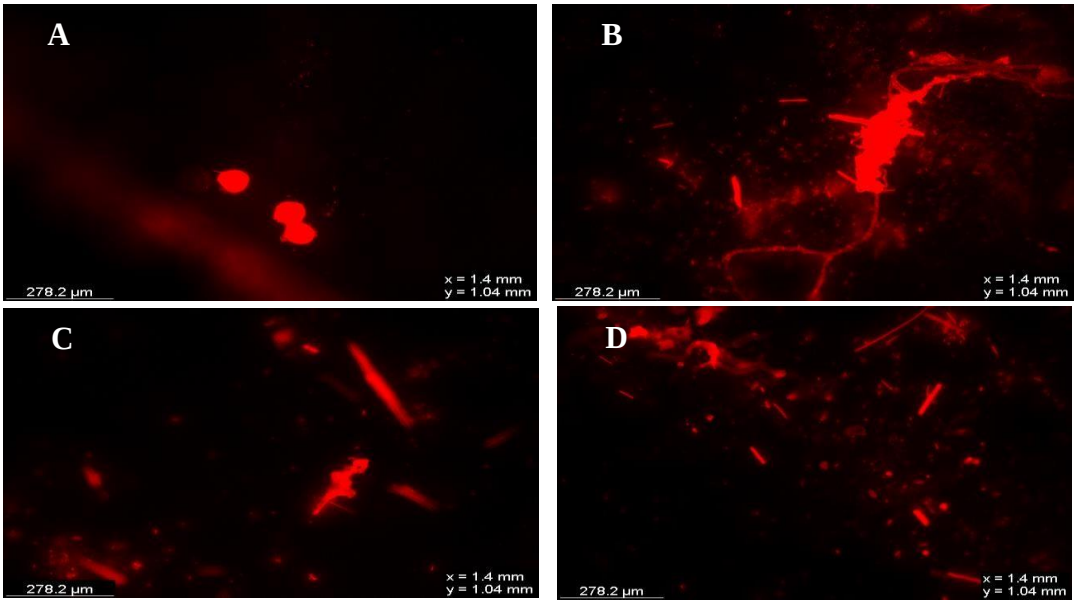

Figure 2.11: Ashton upon mersey water samples; stained with NR, observed under fluorescence microscope at 10X magnification. A) a small bended particle suspected as microplastic B) Suspected plastic particle visualised at different planes. C) long fiber not choosen as microplastic because of living cell like appearance D) suspected plastic fragment.

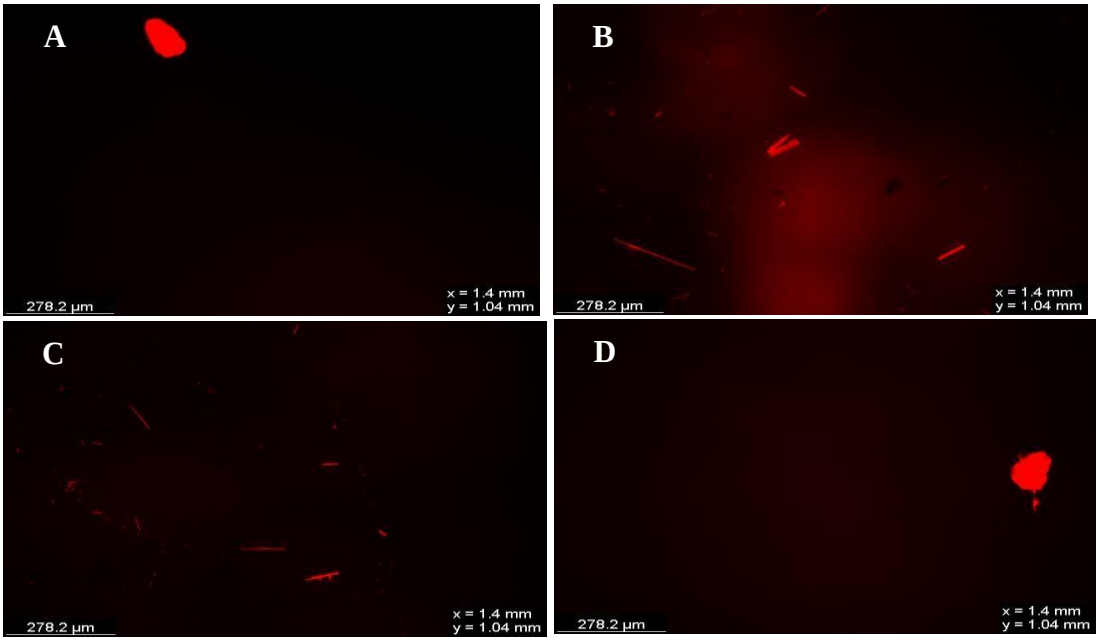

Figure 2.12: Fiddlers Ferry water samples; stained with NR, observed under fluorescencemicroscope at 10X magnification. A) suspected microplastic beads. B,C,D) suspected microplastic fragments and fibres

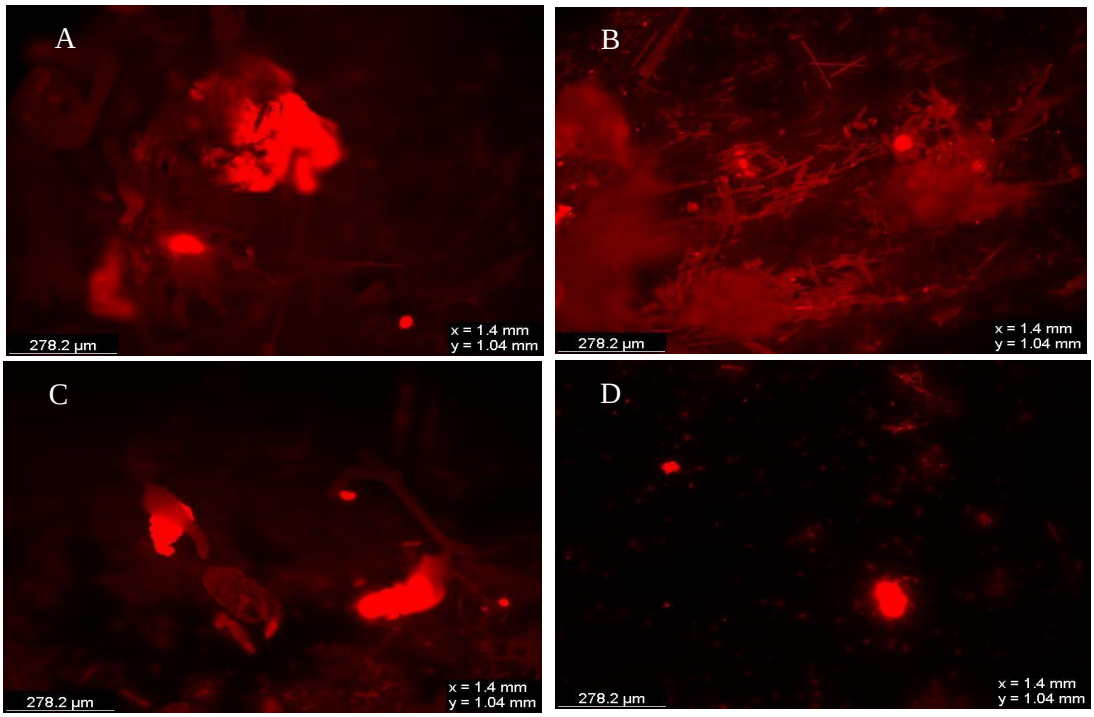

Figure 2.13: Suspected microplastic beads, fragments and fibres in spike Island water samplesstained with NR

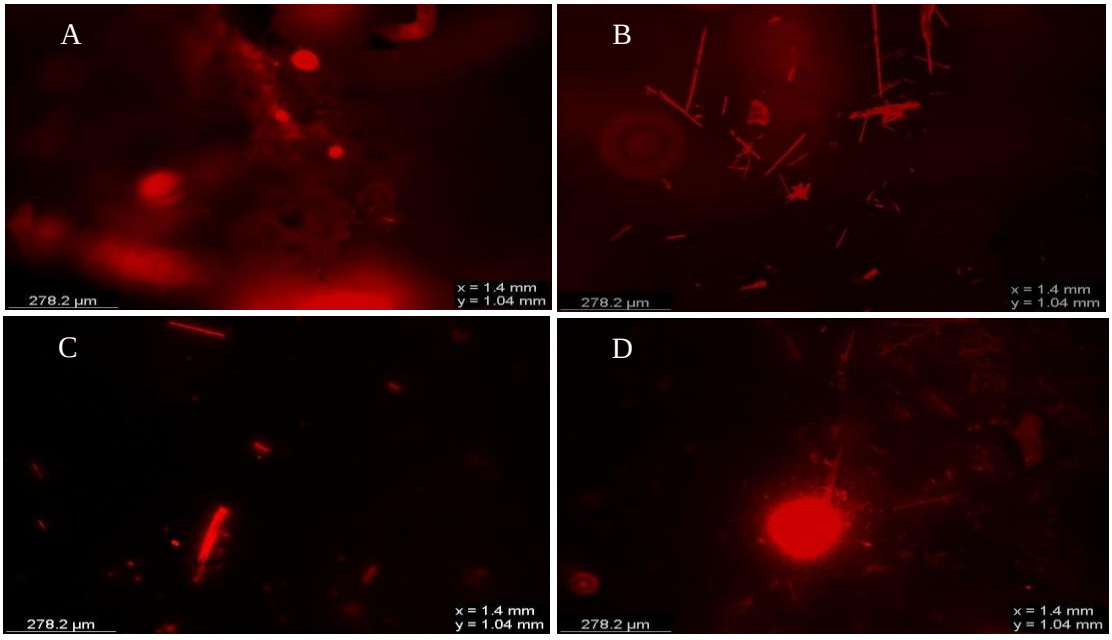

Figure 2.14: Suspected microplastic fibre, fragments and beads in Pickering pasture water samples;dyed with NR stain.

In all five samples, many dark-colored fragments exhibited inconsistent Nile red staining, and possiblymany non-plastic particles were found to fluoresce. Thus, these observations can provide a general estimate of false positive detection and quantification of microplastics in environmental samples. Hence, the technique exhibits considerable limitations to its use for identification and quantificationof microplastic which has not been appropriately validated.

Table 2.6: Microplastics count and size approximation in river water sample (five selected site), stained withNR dye.

Sample (200ml)	Microplastic count						~ size (um)		
	Beads/pellets	Fragments	Fibers/lines	Others	Total	Particles/m^3	<100	100-200	>200
Ashton Upon Mersey	2	0	15	2	19	190	3	1	15
Fiddlers Ferry	4	0	7	0	11	110	9	1	1
Spike Island	2	3	8	0	13	130	7	5	0
Otters pool	2	1	28	0	31	310	3	25	3
Pickering Pasture	1	4	11	0	16	160	0	8	4

In most of previous studies, the validation of NR staining as method of Microplastics identification showed varying results. While some studies concluded it as a reliable method for identification and quantification (Maes et al., 2017; shim et al., 2016), some have reported its limitations as a reliable technique (Stanton et al.,2019).

In light microscopy, blue, purple, black and red fibers were observed which were seen stained with NR dye in fluorescence microscope. This finding is not in accordance with shim et al., 2019 in which they stated that no colored fibers picked up the stain and give fluorescence. But it does not necessarily indicate these stained particles are of plastic origin since only chemical or elemental analysis such as micro-raman or FTIR spectroscopy gives a valid result about the particle identity. However, the staining technique could possibly narrow down the quest for microplastic particle hunt in complex samples and hence make further analysis less laborious.

2.3.4. Micro-Raman Spectroscopy

Most of the particles suspected as microplastics in initial analysis i-e light microscopy and NR staining, were determined to be non-plastic in nature under micro-raman analysis.

The spectral peaks indicated that some particles that were selected as suspected micro/nanoplastic particles in light microscopy were determined to be metallic in nature. Identified particles were Titanium dioxide, Calcite and quartz. This could be implied that due to sample complexity, coenriched particles dominate the raman spectra and suppress the microplastic particles. Some of the peaks indicated the presence of cellulose particles. The cellulose content was potentially originated from the filter papers used for sample filtration as indicated by spectra obtained from blank filter paper. However, one particle was confirmed as Polyethylene tetraphalete (PET) in

nature. The presence of this microplastic particle indicates the river water samples do have microplastic contamination. However, it requires more experiments with more samples to reproduce the results.

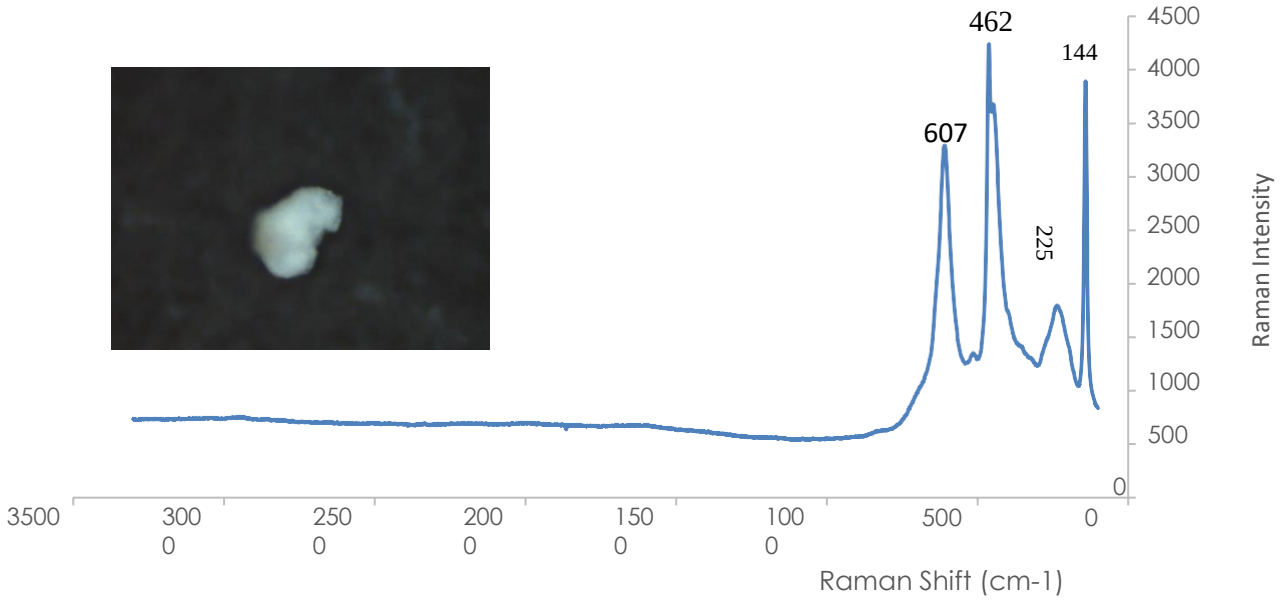

Figure 2.15: Raman spectra of particle identified in Ashton upon mersey sample (Area 3) , identified as Titanium dioxide; Microscopic image of the identified particle

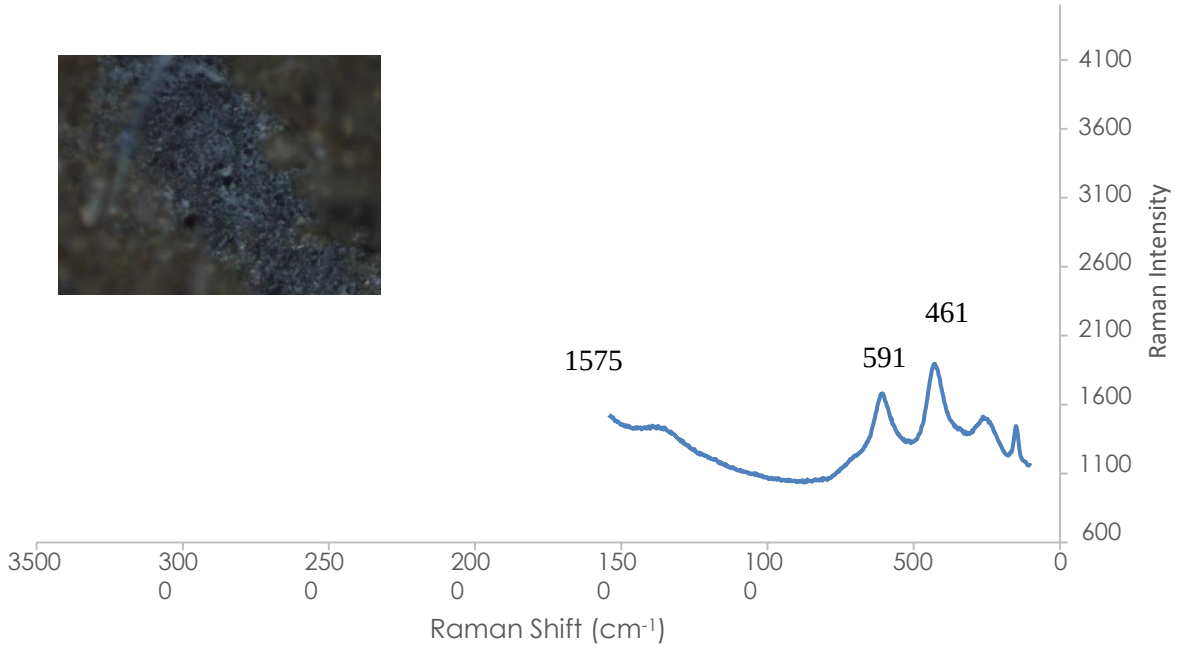

Figure 2.16; Raman spectra of particle identified in Fiddlers Ferry sample (Area 3) ,identified as Titanium dioxide; M:croscopic image of the identified particle

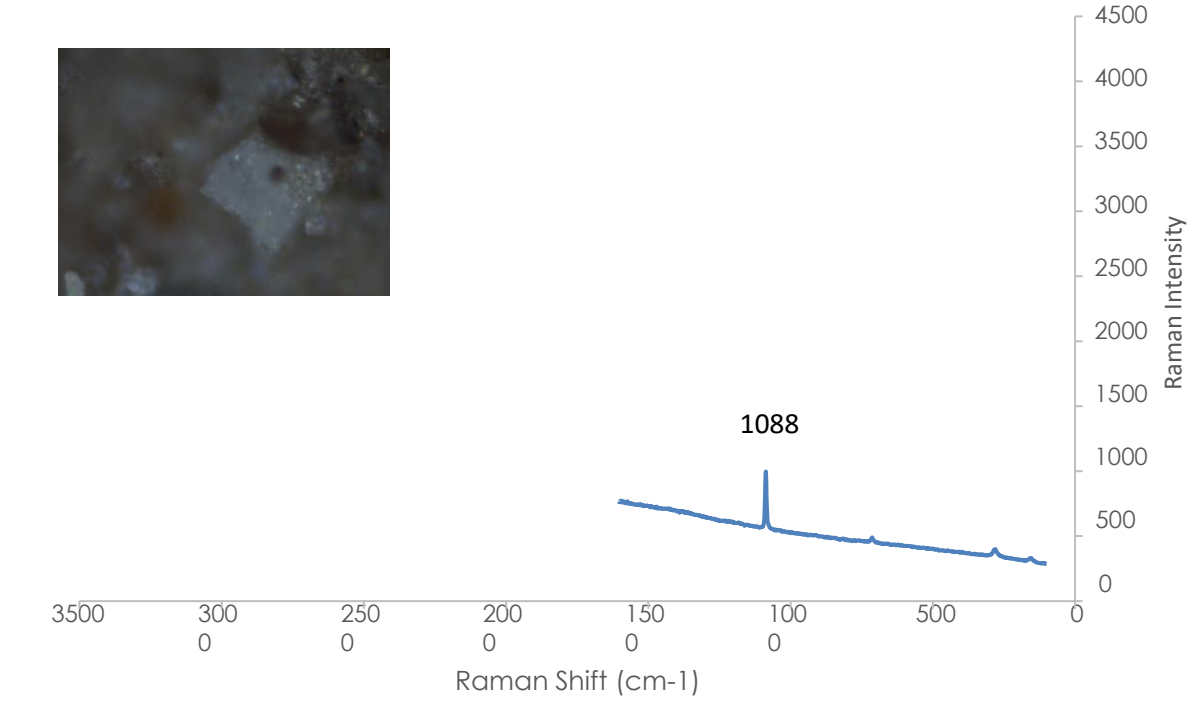

Figure 2.17: Raman spectra of particle identified in Pickering Pastures sample (Area 1) ,identified as Calcite; Microscopic image of the identified particle

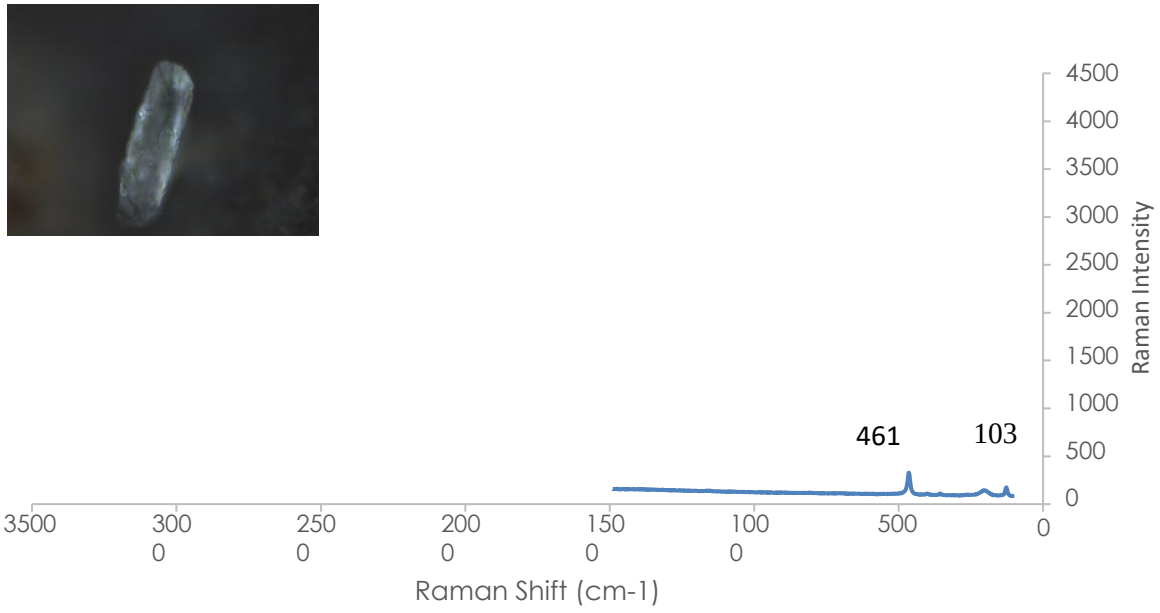

Figure 2.18: Raman spectra of particle identified in Pickering Pastures sample (Area 3) , identified asQuartz; Microscopic image of the identified particle

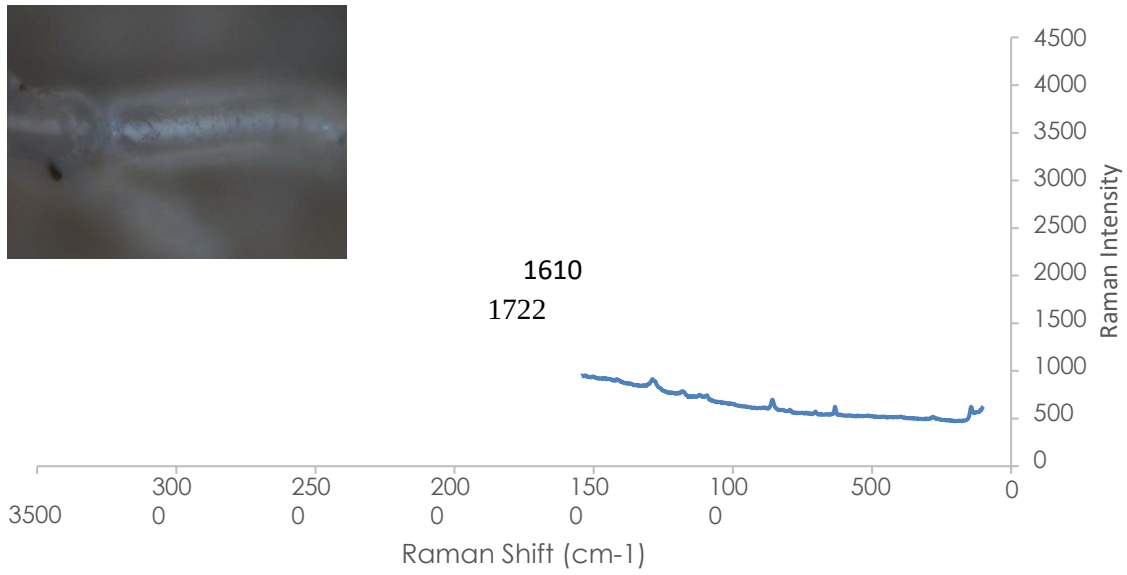

Figure 2.19: Raman spectra of particle identified in Fiddlers Ferry sample (Area 3) , identified asPET; Microscopic image of the identified particle

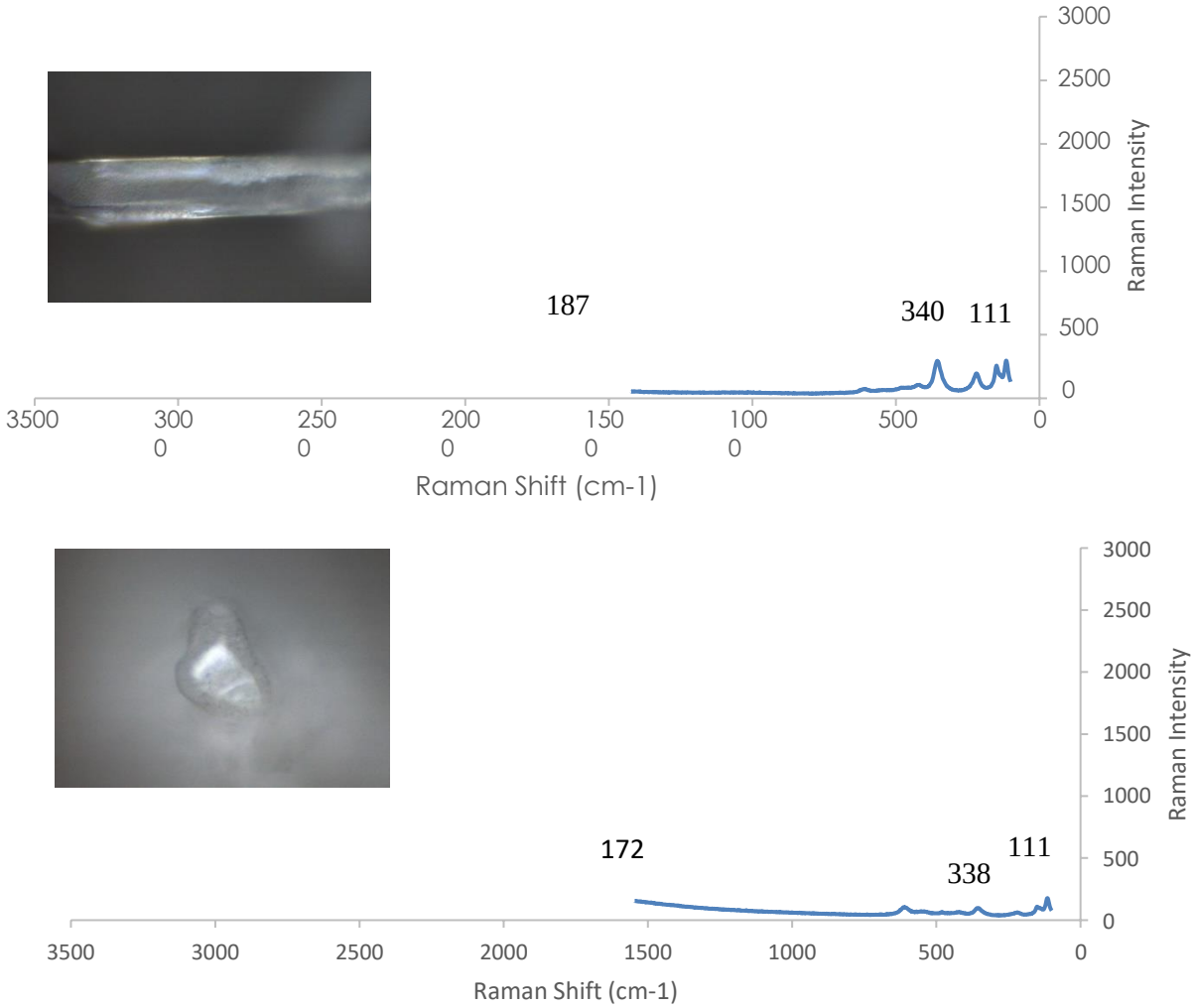

Figure 2.20,2.21: Raman spectra of Ashton upon Mersey NR dyed sample, representing peaks for Nile

2.4. Discussion

The use of plastic products has increased considerably in recent decades to meet the exploding resource demand. Nevertheless, the long-term implications of this surge in plastic consumption pose a significant concern. Contamination of oceans and rivers with plastic pollutants produce deleterious impacts on marine and aquatic biota. The literature provides evidence of many harmful effects of plastics at micro and macro levels (Cole *et al*., 2015; Cole *et al*., 2013; Ogonowski *et al*., 2016; Rist *et al*., 2017). The interaction of microplastic particles with the charged particles such as cations or heavy metals occurring in aqueous environment results in formation of hetero aggregates. Aggregate formation plays a crucial role in functionality and chemistry of microplastic particles. Therefore, these parameters may impact the activity, toxicity, fate, and transport of the microplastic particles in the aquatic environment (Singh et al., 2019).

In recent years, there has been a growing emphasis on conducting research to examine environmental samples for microplastics quantification, assessment of their negative impacts on living organisms in presence of co-pollutants and explore their potential degradation mechanisms. Recent advancement in quantification techniques have make it possible to assess the nature and type of microplastic particles for further investigations, such as Fourier transform infrared spectroscopy (FTIR), Raman spectroscopy and Mass

spectrometry. But there are certain limitations to these methods and hence validation of more suitable analytical methods is required. In this study, established techniques were evaluated for detection and identification of microplastics in freshwater samples. Microscopic analysis indicated the presence of many microplastic particles. Based on their shapes, observed microplastics were classified as fragments, fibers, pellets and those which were difficult to classify were classed as others. This classification could be of importance as it might indicate the parent materials of the microplastics found in samples. (Zhang et al., 2018). Fibers were most abundant in all studied samples (Figure 3.2). These fibers probably originated from fishing nets or textiles. The spillage of drainage from waste water treatment plants into river Mersey seems to be most obvious source of MP pollution in the studied river. Since the untreated waste water have high content of laundry waste water, presence of fibers was expected to be high (Dris et al., 2016; Salvador Cesa et al., 2017, Shim et al., 2016). The samples stained with NR dye yielded identical results. There were even more number of particles stained by the NR dye as compared to those observed in light microscopy. Micro-Raman analysis was very specific in terms of polymer identification. In microscopic analysis, the particles which appeared to be dark colored, standout of background and have properties used for selection of particles as microplastics in literature, chemical identification by micro-Raman spectroscopy described them as non-plastic. These findings are in accordance with those concluded by Blair et al., 2019 which reported that results of visual inspection of particles differ from those obtained from

chemical/elemental analysis. According to a study conducted by Blair et al. in 2019, microscopic examination alone can lead to the misidentification of microplastics due to the presence of physical similarities but varying elemental compositions among micro-sized particles.

2.5. Conclusion

The current investigation has revealed that the existing methodologies used to measure microplastic exposure in different environmental contexts may not provide accurate information. This highlights the urgent need to develop novel approaches that can better capture the true levels of microplastic exposure.

The current investigation has identified limitations in the existing methodologies used to measure microplastic exposure. These methodologies often rely on visual identification or chemical analysis, which may not capture all types and sizes of microplastics present in the environment. Additionally, the methods used to collect samples may introduce biases and inaccuracies, leading to underestimation or overestimation of microplastic levels.

To address these limitations, it is necessary to develop novel approaches that can improve the accuracy of microplastic measurements. This may involve the use of advanced imaging techniques, such as electron microscopy or spectroscopy, to identify and quantify microplastics more precisely. Furthermore, the development of standardized protocols for sample

collection and processing will help minimize biases and ensure consistency in data collection across different studies.

Enhancing existing methodologies is also crucial in obtaining more accurate information about microplastic exposure. This may involve refining the current techniques used for visual identification or chemical analysis to improve their sensitivity and specificity. Additionally, incorporating multiple analytical techniques and cross-validation of results can help validate the findings and increase confidence in the data obtained.

By developing novel approaches and enhancing existing methodologies, researchers will be able to obtain more accurate information about the levels of microplastic exposure in various environmental contexts. This, in turn, will enable a better understanding of the potential effects of environmentally realistic exposure levels on organisms. Such knowledge is essential for developing effective strategies to mitigate the impacts of microplastic pollution and protect the health of ecosystems and human populations.

CHAPTER 3

COMBINED EFFECT OF CRYOGENICALLY MILLED MICROPLASTICS (PET AND HDPE) AND METALS (PB^{2+} AND FE^{2+}) ON *DAPHNIA PULEX* PHYSIOLOGY AND EXPRESSION OF FUNCTIONAL GENES.

3.1. Introduction

3.1.1. Microplastics

Micro plastics take different routes to enter water bodies that include both fresh water and sea water (Duis & Coors 2016) Currently, microplastics are ubiquitous, in streams, river mouth, on seashores, the ocean surface or in the water columns, and on the seafloor (GESAMP 2015). The pervasive nature of the micro or nano plastics in the environment makes their interaction with water biota inevitable. The bio availability of micro or nano plastics greatly depends on their size, shape, and density (Wright *et al*., 2013). According to Ocean Plastics Lab 2018, approximately 1400 marine species are known to be exposed to marine plastic pollutants via different ways with entanglement and ingestion being the most common types of interaction between water biota and micro/nano plastics (Gregory, 2009). The possible effects of micro/nanoplastics on living organisms present in water include pathological stress, reproductive complications, changes in enzymes activities, reduced growth rate, and oxidative stress (Besseling *et al*. 2014; Sutton *et al*. 2016). Relatively smaller particles (<100 nm) may pose greater consequences when being ingested, because they may end up in the tissues or even inside the cells (Lusher, 2015). Moreover, the time a particle spends inside the body (i.e., the retention time) is crucial for estimating chemical damage within the body.

Even the aquatic primary producers called phytoplankton such as algae and

mosses have also been shown to interact with microplastic pollutants. Bhattacharya *et al*. (2010) have reported in their research that nanosized plastic particles can be adsorbed by a green algae (*Scenedesmus* spp.), resulting in reduced photosynthetic activity. Some phytoplankton species can form aggregates with Microplastics. Long *et al*., (2015) demonstrated that the phytoplankton *Rhodomonas salina* has a inclination to adsorb more microplastic forming aggregate as compared to *Chaetoceros neogracile*. More serious effects are addressed in a recent study Kalcikova *et al*., (2017) with a freshwater organism duckweed with sharp polyethylene microplastics from exfoliating cosmetic products. The interaction results in reduced viability of the root cells of the duckweed (*Lemna minor*), which adversely affects their development.

Despite this, a significant number of publications have reported detrimental effects of micro and nano plastic pollution on aquatic as well as coastline living organisms. For example, in a study, crustaceans when exposed to plastic particles shown to have adverse effects. Their exoskeleton was affected and with the increase in exposure time, increased mortality and reduced growth was observed. (Aljaibachi and Callaghan, 2018). To date, many aquatic organisms have been explored to evaluate the toxic effects of micro and nanoplastics under lab conditions. the major detrimental effects observed in different researches include abnormal embryonal development (Lee *et al*., 2013; Jeong *et al*., 2017), decreased lipid droplet storage (Cui *et al*., 2017),

decreased feeding rates (Cole *et al*., 2015; Cole *et al*., 2013; Ogonowski *et al*., 2016; Rist *et al*., 2017), energy depletion (Cole *et al*., 2015), decreased survival (Au *et al*., 2015; Manfra *et al*., 2017), reduced growth (Aljaibachi and Callaghan, 2018; Au *et al*., 2015; Besseling *et al*., 2014; Jeong *et al*., 2016; Redondo- Hasselerharm *et al*., 2018; Ziajahromi *et al*., 2018), altered reproduction (Au *et al*., 2015; Besseling *et al*., 2014; Cole *et al*., 2015; Cui *et al*., 2017; Jeong *et al*., 2017; Jeong *et al*., 2016; Lee *et al*., 2013; Ogonowski *et al*., 2016; Ziajahromi *et al*., 2017), malformations (Besseling *et al.,* 2014), delay in molting (Jeong *et al*., 2017), abnormal swimming behavior (Rehse *et al*., 2016; Ziajahromi *et al*., 2017) and damaged intestinal microvilli (Chae *et al*., 2018).

3.1.2. Choice of model organism '*Daphnia pulex*

Since the present study aims to evaluate the freshwater environment for microplastic contamination and assess their impact on living organisms inhabiting the freshwater habitat, a freshwater crustacean **'*Daphnia pulex*'** was chosen as a model organism to conduct the toxicity experiment on, using the cryogenically milled microplastics (PET and LDPE).

Daphnia have proven to be a valuable research tool for various reasons. Due to their wide abundance in freshwater habitats, daphnia serve as an important link between primary producers and consumers of higher tropical levels (Yin et al.,2023). Hence the impact of contaminants on daphnia population can extrapolate their impact on higher organisms. Moreover, the

short life span, easy mode of reproduction, and sensitivity to environmental contaminants makes them a convenient to use biomaterials for studying the toxicity and ecological risks of microplastics. To date, a lot of research publications have reported the negative impact of different types of microplastics alone or in combination with other contaminants such as heavy metals or certain additives such as BPA, on different daphnia in various aspects. For instance, some studies have focused on individual-level impacts on daphnia population following microplastic exposure in the laboratory, such as mortality and sublethal impacts (e.g., altered physiological, morphological, and developmental traits) (Rehse et al., 2016, Liu et al., 2021).

3.1.2.1. *Daphnia* modes of microplastics ingestion:

The ingestion of microplastics immediately starts to affect the daphnia's life cycle. As soon as the microplastic particles are inside the daphnia body, they interfere with the biological functions and results compromised life quality or death of organism. There are possibly two modes of microplastics ingestion in daphnia i) **direct ingestion**, which means the daphnia actively uptakes the microplastic particles as food or ii) **Indirect ingestion**, is the process of unconsciously ingesting microplastics that exist on the surface of or inside its food (e.g., algae) (Yin et al., 2023). The active or direct ingestion of microplastics by daphnia occurs because for daphnia it is difficult to distinguish between food particles and the microplastics when they coexist in the natural environment. In both cases, the presence of microplastics in the

medium makes their ingestion inevitable. However, the quantity of microplastics being ingested or the extent of the toxicity they impose after being ingested strongly depends on many other factors which will be discussed in sections below. Aljaibachi and Callaghan (2018), reported the selective feeding behaviour of daphnia where in absence of any other food source, daphnia ingest microplastics as food whereas the microplastic ingestion is strongly reduced in presence of other food particles such as microalgae. Hence the microplastics competes with the natural food sources. Where the direct ingestion is restricted in presence of preferred food source, probability of indirect or unconscious ingestion is higher.

3.1.2.2. Factors affecting microplastic ingestion.

With the advancements in microplastics research, it is concluded that the ingestion of microplastic particles depends on various factors. These factors include i) size of microplastic particles ii) chemical nature or type of microplastic particles iii) concentration of microplastics in the environment and iii) the duration of exposure (Yin et al 2023). Research by Kokalj et al. (2018) concluded that the smaller sized microplastic particles are more likely to be ingested and distributed inside daphnia body as compared to larger microplastic particles. Furthermore, it has been observed that the smaller microplastic particles exhibit an extended duration of stay within the daphnia organism (Rosenkranz et al., 2009). The amount of microplastic particles been ingested is also strongly correlated to the exposure time and its concentration

in the surrounding environment. A study by Rist et al., 2017 concluded that when daphnia are exposed to a certain concentration of microplastics for longer duration, the rate of ingestion reaches saturation level and that if the concentrations are higher, it takes less time to reach the saturation level.

After ingestion, these microplastics can accumulate in different organs and alter the organism's normal functionality. The first destiny for these microplastics in daphnia's body is the intestinal tract where its accumulation clogs the gut and thus depleting the nutrients required for organisms' survival (Eltemsah and Bøhn, 2019). Smaller sized polystyrene microplastics have been reported to enter the ovaries and brood chambers, which was found to have negative impacts on organisms' survival and reproduction (Brun et al., 2017, Cui et al., 2017). Nevertheless, majority of the research conducted on this matter has been confined to a single form of microplastic, specifically 'polystyrene', and has also been limited to a specific model organism. Given this, the current investigation seeks to assess the influence of PET and LDPE on the physio-chemical and genetic expression of the model organism 'Daphnia pulex,

3.1.3. Preparation of Reference material in lab

The choice of microplastic type i) Polyethylene Terephthalate and ii) Low density Polyethylene, for this study was based on its relative abundance in the targeted freshwater environment.

3.1.3.1. Polyethylene Terephthalate

Polyethylene terephthalate commonly known as PET is one of the most common and important polyester. It is produced by terephthalic acid and ethylene glycol when they undergo polycondensation reaction as shown in Figure 3.1.

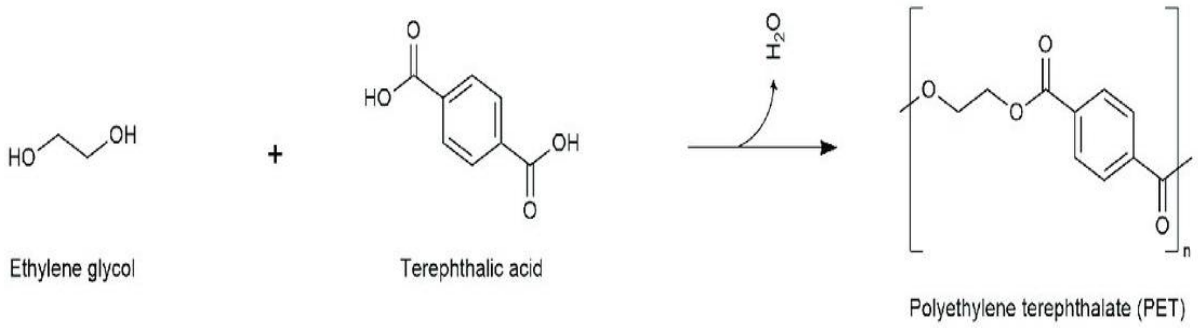

Figure 3.1: Polycondensation reaction between ethylene glycol and terephthalic acid to form polyethylene terephthalate (PET) monomer, with the release of H_2O molecule.

The characteristic of being transparent, amorphous, and thermoplastic makes it an excellent choice for wide use in food packaging. One of the most common uses of PET is to fabricate carbonated beverage bottles because it has high strength and toughness, good abrasion and heat resistant, good chemical resistance, and excellent dimensional stability as well as no penetration or exchange of gases (Plastics and Sustainability (Sin & Tueen, 2023). Another important application of this polyester is in the textile industry. Track suits and bedding made of PET are used in large numbers.

However, the PET is a nondegradable polyester and hence PET waste has

become a major portion of plastic pollution (Kim et al., 2020). Various research conducted on life cycle assessment of PET packaging and investigations on assessment of its environmental impact has revealed that manufacturing process of PET contributes to 49 environmental impact categories. Among these categories, global warming potential, human toxicity, and marine aquatic ecotoxicity are the three highest impact categories (Lee Tin Sin, Bee Soo Tueen, in Plastics and Sustainability, 2023). These mentioned environmental impact of PET are due to a very large amount of CO_2 released and use of toxic chemicals during the polymerization process. The presence of benzene ring in the PET polymer is of very much concern. Benzene is known to be a very toxic molecule. According to 'a consolidated short review of human and animal studies' by Haseeb Ahmed khan (2017), Benzene is an established human and animal carcinogen. Exposure to benzene has been associated with leukaemia in humans and several types of malignancies in animals (James Huff, 2013). All these factors made our choice for this polymer type to assess for its toxicological environmental impact on freshwater model organism '*Daphnia pulex*'.

3.1.3.2. Low density Polyethylene (LDPE)

Low density polyethylene commonly known as LDPE is a widely used thermoplastic made by the free radical polymerization of monomer 'ethylene' as shown in figure 3.2.

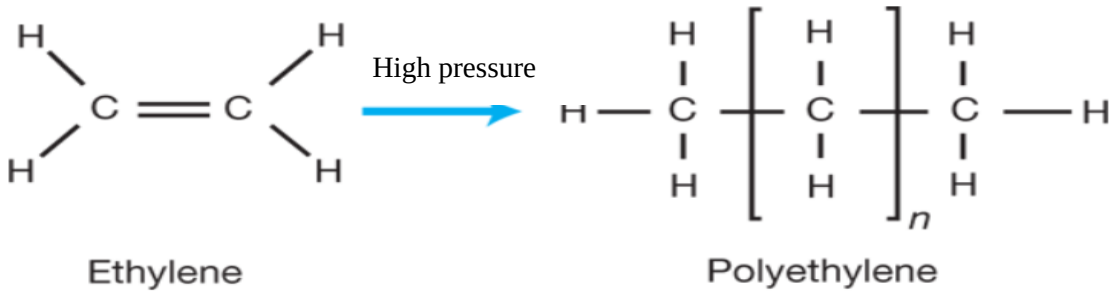

Figure 3.2: Polycondensation reaction between ethylene glycol and terephthalic acid to form polyethylene terephthalate (PET) monomer, with the release of H_2O molecule (AIChE, The global Home of Chemical Engineers).

The high degree of short and long branching in the polymer as well as low instantaneous dipole–dipole interactions, resulting in less intermolecular forces makes it a very low dense polymer giving it a characteristic property of being very flexible and low dense. The wide application of this polymer is in plastic shopping bags, manufacture of disposable containers, squeeze bottles and many more. Hence, LDPE enter waste stream through different sources that includes packaging materials, Single-use items like disposable cups, lids, straws, and cutlery are often made from LDPE, Plastic Film and Sheets and construction and building materials.

Although LDPE has been ranked as least hazardous based on monomer hazard classification, among the list of virgin plastics, however its wide abundance in the waste stream and impact of other environmental factors on the plastic polymers when in waste stream makes its assessment for

freshwater biota toxicity inevitable. Several studies have reported the negative impact of LDPE microplastic exposure on terrestrial as well as aquatic invertebrates. For instance, Lwanga *et al* (2016) have reported the reduced survival and growth rate of earthworm *Lumbricus terrestris* after long term exposure to polyethylene. Decreased reproduction and avoidance behaviour of springtail *Folsomia candida* was observed by Chen *et at* (2020) at the concentration of 1.5 g/kg LDPE after exposure 28 days along with morphological damage and antioxidant response of studied organism.

Hence LDPE and PET were chosen to conduct the toxicity assay on *Daphnia pulex*.

3.2. Materials and Methods

3.2.1. Preparation of reference material

PET and LDPE granules were purchased from Sigma Aldrich, UK. To produce microplastic particles of a desired size range from about 30 to 200 µm, a combination of cryogenic milling and different sieving steps were done. To this end, 20 g of PET and LDPE granulate was cryo-milled separately using a Retsch MM400 mixer mill. Liquid nitrogen was used to prevent thermal degradation during the milling process by giving cryo effect to the PET and LDPE granules before grinding. The granulates were than ground through 15 cycles of 3 min with a cooling period of 5 min in between each step. After milling, the PET and LDPE powder produced was transferred into a separate glass beaker with a glass cover to prevent contamination by airborne

particles.

3.2.2. Particle characterization

To determine the particle size of cryomilled PET and LDPE particles, laser diffraction particle size analyzer LA 960, Horiba scientific was used. Briefly, 1 mg of cryomilled powder was added to the sample reservoir. To avoid particle aggregation, the sample was stirred, and ultrasonic energy was used. Three measurements were recorded for each microplastic (PET and LDPE) and graph was plotted in Microsoft Excel.

Scanning electron microscopy SEM/EDX analysis was performed for each of the cryomilled microplastic type to visually evaluate the impact of cryomilling on particle surface morphology. SEM/EDX allowed many potential microplastic particles to be screened in a relatively short time. The analyses were conducted using JSM-6480LV Scanning Electron Microscope (JEOL Ltd., Tokyo, Japan) and high-resolution images useful to the in-depth observations of the morphological details were obtained.

3.2.3. Pb^{2+} and Fe^{2+} metal adsorption on PET and LDPE microplastics

Stock solutions of concentration 5mg/l (in bottled mineral water) of both PET and LDPE powder were prepared separately. For each of the stock solutions, a working concentration of 1mg/l was prepared using formula M1V1 = M2V2. Similarly, the stock solutions of $PbCl_2$ and $FeCl_2$ were prepared separately by dissolving 1mg of the respective metal salt in bottled mineral water to get a

final concentration of 1 mg/l salt solution. Equal volume of both stock solutions for PET and LDPE and the metals were mixed in separate combinations and let stand for 48 hours (shaking the solutions after intervals) to allow the maximum interaction of metals and the microplastics. After 48 hours, solutions were centrifuged for 5 mins at 3600 rpm, and the solid phase (the microplastic with potentially adsorbed metals) was separated and dried completely in glass petri dish. The dried microplastic was then evaluated for adsorption capacity for the respective metals using XRF. X-ray Fluorescence (XRF) is an analytical technique that uses the interaction of X-rays with a material to determine its elemental composition.

3.2.4. Bioassay

Experimental conditions were determined for sub-lethal exposure. Initial feeding assays at 5mg/L of MP were lethal, dilution to 1mg/L was the highest concentration were daphnia survived.

The fresh culture of *Daphnia* was purchased from Blades Biological Ltd. UK. Culture was reared in optimized lab conditions to be used for subsequent bioassay experiments. Briefly, *Daphnia* culture was maintained at water temperature of 18 ± 1 °C, photoperiod of 16 h light: 8 h dark. Glass bottled mineral water was used as culture medium. Food was *Chlorella vulgaris (purchased from blades biological Limited UK)* provided every day from Monday to Friday, 3×10^5 cells/ml (Calculated using hemocytometer).

For the bioassay, the following treatment groups were used to expose

daphnia pulex for period of 7 days.

Group 1 Low density polyethylene LDPE single,

Group 2 Polyethylene Terephthalate PET single,

Group 3 Lead ion Pb^{2+} single,

Group 4 Iron ion Fe^{2+} single,

Group 5 LDPE+Pb^{2+} ,

Group 6 LDPE+Fe^{2+}

Group 7 PET+Pb^{2+},

Group 8 PET+Fe^{2+},

Group 9 LDPE+PET+Pb^{2+}+Fe^{2+}.

Normal Growth Media (NGM) was used as positive control. The bioassay weas started with juvenile daphnia (first brood, 48h-72h old). Ten juvenile Daphnia were exposed to each treatment group in 500 mL glass beakers with 300 mL of test medium. Beakers were covered but allowed air changes. The test medium was refreshed every 24 h, the exposure period was 7 days. Organisms were exposed with a final concentration of 1mg/l of the cryomilled PET and PE particles in above mentioned treatment groups.

The following physiological parameters were studied over a period of seven days as effect criteria.

I. **The mean of somatic growth** per daphnia (Growth indicators taken as body length and body width) was measured under Leica DM750 light microscope

at 20X magnification. Images were captured with the attached camera and *imageJ* software was used to record the measurements.

II. **Mortality rate** (No. of Live organisms/total number of organisms * 100) was recorded by counting the number of live moving daphnia in each treatment group.

III. **Beats per minute (BPM)** were recorded by visually counting the number of times heart beats in 10 seconds and then was calculated for one minute by multiplying the recorded number with 6.

IV. **Reproduction** (number of embryos in brood chamber) was recorded by counting the number of embryos in the brood chamber.

Statistical analysis using IBM SPSS was performed to determine the significance of the findings. A general linear model was applied to analyse body length, body width and heart rate of daphnia. The results with p-value less than 0.05 were taken as significant.

3.2.5. Differential RNA expression analysis

RNA seq analysis was performed on high quality total RNA samples extracted from all the treatment groups. The RNA extraction was done using the RNA Aqueous Micro kit (Thermo fisher Scientific).

In each treatment group, 20-25 Daphnia were exposed to the experimental conditions and then were harvested after 48 hours. The harvested Daphnia were kept separately for each treatment group in RNA later (Thermo fisher Scientific) for 24 hours at $4^{O}C$. After that the Daphnia from each treatment

group (20-25 Daphnia) were separated from RNA later and were homogenized using glass pestle in a glass tube to continue the extraction procedure using RNA Aqueous Micro kit (Thermo fisher Scientific). The extracted RNA was sent to a sequencing service (Azenta) for RNAseq sequencing. The mRNA focussed sequencing libraries were prepared and quality controlled was done by the sequencing service and then sequenced on an Illumina platform, aiming for paired end 150bp reads to give 30 million read pairs. **Fastq** files provided by the sequencing service were analysed using a workflow in 'R' version 4.3.1.

Using 'R', the Sequences were sampled, quality controlled and viewed using the ShortRead library (Morgan et al., 2009). Morgan et al (2009) stated that "ShortRead is a Bioconductor package for input, quality assessment and exploration of high-throughput sequence data." The genome file for the *Daphnia pulex* genome (*Daphnia pulex* (Common water flea, KAP4) - "GCF_021134715.1_ASM2113471v1_genomic.fna" and the gtf annotation file were downloaded from ncbi. A gapped and split index was constructed for the genomic file using the Rsubread library (Liao Y, Smyth GK, Shi W (2019). "The R package Rsubread is easier, faster, cheaper and better for alignment and quantification of RNA sequencing reads." *(Liao, Smyth & Shi, 2019)*. Reads were mapped to the NCBI genome file index using the align function in Rsubread. Reads mapping to genes were counted using the 'featureCounts' function in Rsubread. Reads were converted into counts per million (cpm)

and a TMM normalisation was applied. The Normalised reads were analysed using EdgeR.

There were 23 samples (out of 30 total samples; 10 treatment groups in three replicates) with usable data. 7 samples could not produce useful information because of poor sample quality with less than required amount of RNA extracted and hence could not produce the RNA library. The remaining data was then analysed with four 2 level factors (Pb, Fe, PE, PET), with the treatment combinations allowing 2-way interactions for the metals with the plastics to be estimated.

The main effect tests were carried out in a main effect–only model; and then the interactions were added to test the interactions. The Normalized Enrichment Score (NES) derived from the analysis provides insights into whether the genes in a specific pathway are upregulated or downregulated. The NES is a statistical measure that quantifies the degree to which a gene set is enriched in a given dataset. It takes into account both the direction and magnitude of gene expression changes within the pathway. To calculate the NES, the gene expression data is first ranked based on their differential expression between experimental conditions. Then, a running-sum statistic is computed by accumulating the expression changes as the ranked genes progress through the pathway. The NES is obtained by normalizing this running-sum statistic with respect to its mean and standard deviation. A positive NES indicates that the genes within the pathway are enriched in upregulated

genes, suggesting an activation of the pathway. Conversely, a negative NES indicates enrichment in downregulated genes, indicating repression of the pathway. The magnitude of the NES reflects the extent of the enrichment, with larger absolute values indicating stronger enrichment. **GSEA** plots were obtained using gfsea package R was performed which allowed to probe for the effect of selected groups of genes or "pathways". "Oxidative stress", "Reproduction" and "Development" organismal pathways were selected for assessment of toxicity effects. Finally, Heatmaps were produced to visualize the specific genes differentially expressed in each studied pathway. A heatmap is a tool that gives visual representation depicting differentially expressed genes within individual sample groups. It enables the identification of altered gene expression having statistical significance among hundreds to thousands of genes, each of which is correlated with various exposure settings. In the heatmap produced, colours were employed to represent diverse sets of values using a continuous colour map (Carroll et al., 2020). The x-axis of the heatmap represents the treatment groups, while the y-axis, shows the individual gene IDs differentially expressed in the treatment groups. The colours in the heatmap relates to the level of gene expression, ranging from high to low, and are determined by the values within a defined range. In the heatmaps below, downregulation is shown by the green colour whereas the red colour implies upregulation.

3.3. Results

3.3.1. Particle characterization

The size distribution of cryogenically milled PET and LDPE microplastic particles is shown in Figures 3.3 and 3.4.

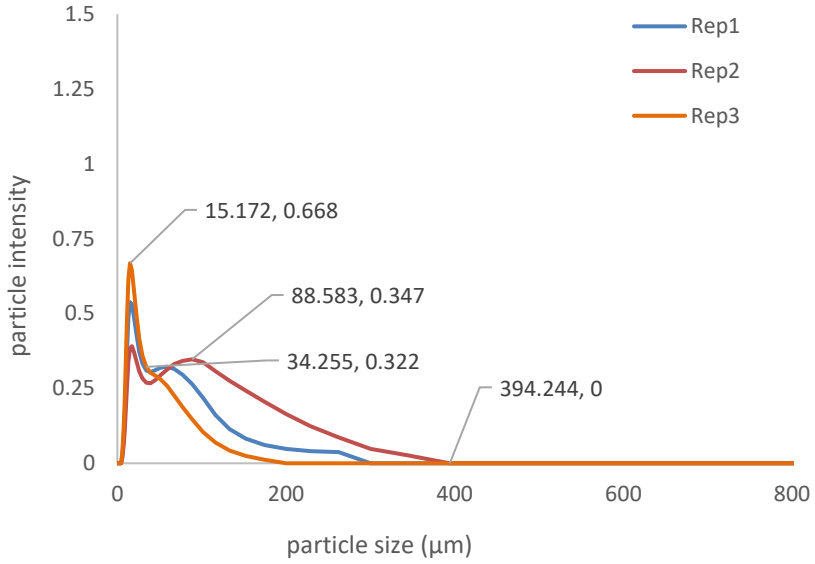

Figure 3.3: Particle size distribution of crogenically milled PET particles; x-axis represents particle size in microns, y-axis represents particle intensity.

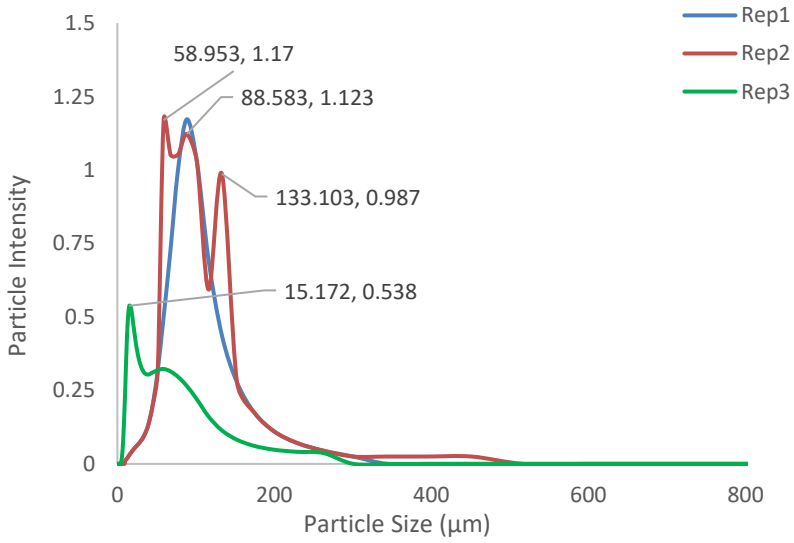

Figure 3.4: Particle size distribution of cryogenically milled LDPE particles; x-axis represents particle size in microns, y-axis represents particle intensity.

The majority of PET microplastic particles fall within the size range of 15–400 µm, with the highest proportion of total mass being accounted for by particles approximately 15 microns in size. Figure 3.3 illustrates that the size range of 60-80 microns contains the second highest number of particles. Conversely, most LDPE particles are concentrated within a relatively narrow size range, with the highest particle intensity observed at approximately 50 microns. In comparison to PET particles, the LDPE particles exhibit a narrower size distribution.

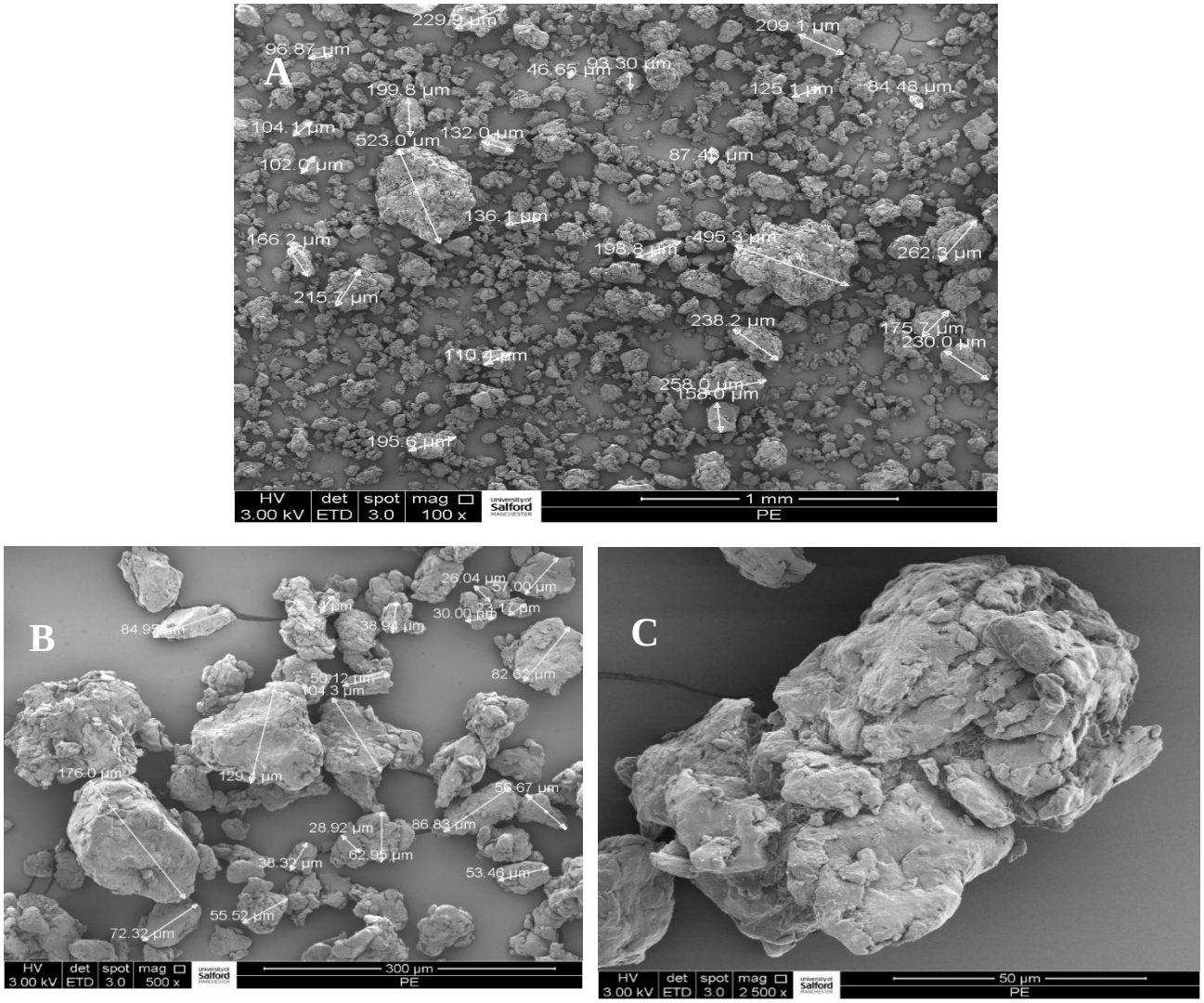

Figure 3.5: Scanning electron microscope images of the cryogenically milled LDPE microplastic particles. Top image shows lower magnification view covering large number of crushed particles. Bottom views show detailed images of crushed particles.

The micrograms obtained through scanning electron microscopy revealed that the particles of both plastic types exhibit irregular shape and surface morphologies.

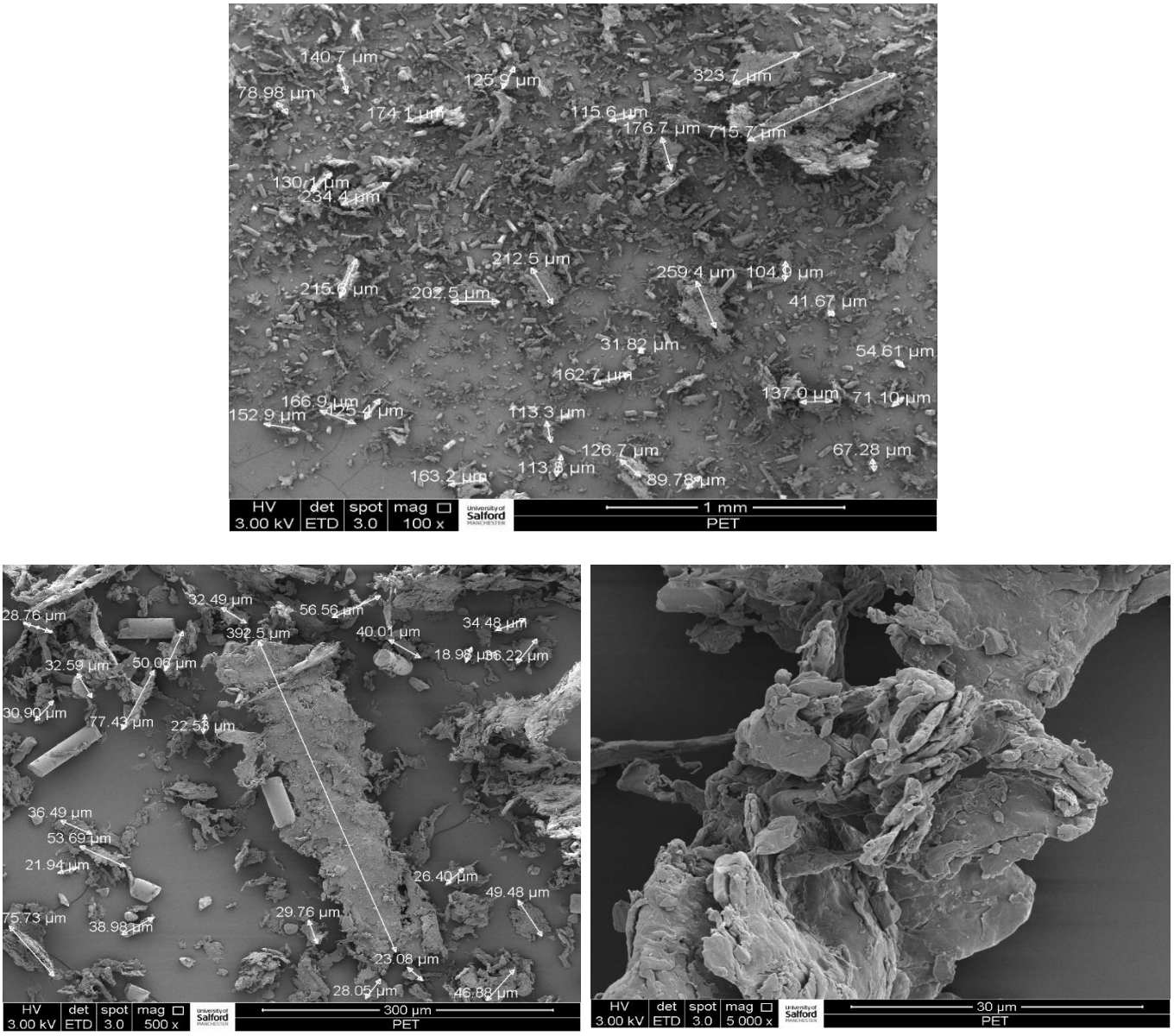

Figure 3.6: Scanning electron microscope images of the cryogenically milled microplastic particles. Top image shows lower magnification view covering large number of crushed particles. Bottom views show detailed images of crushed particles.

In more detail, when PET (polyethylene terephthalate) particles were subjected to cryogenic milling, which involves grinding the particles at extremely low temperatures, they showed additional variations in both size and shape as well as surface morphologies. This means that the resulting milled particles were not only smaller, but they also had different shapes compared to the initial PET particles. Furthermore, the milling process itself led to the formation of smaller particles when compared to LDPE (low-density polyethylene). This suggests that cryogenic milling is an effective method for reducing the size of PET particles then LDPE.

3.3.2. Pb^{2+} and Fe^{2+} metal adsorption on PET and LDPE microplastics

The results from X-ray fluorescence analysis indicated that both selected metals, namely Pb^{2+} and Fe^{2+}, showed the ability to adsorb cryogenically milled microplastics. However, it was observed that PET microplastics had a higher affinity for adsorption compared to the LDPE. The highest adsorption capacity was recorded for Pb^{2+} on PET microplastic, at a concentration of 1573 ppm, as shown in Figure 3.7.

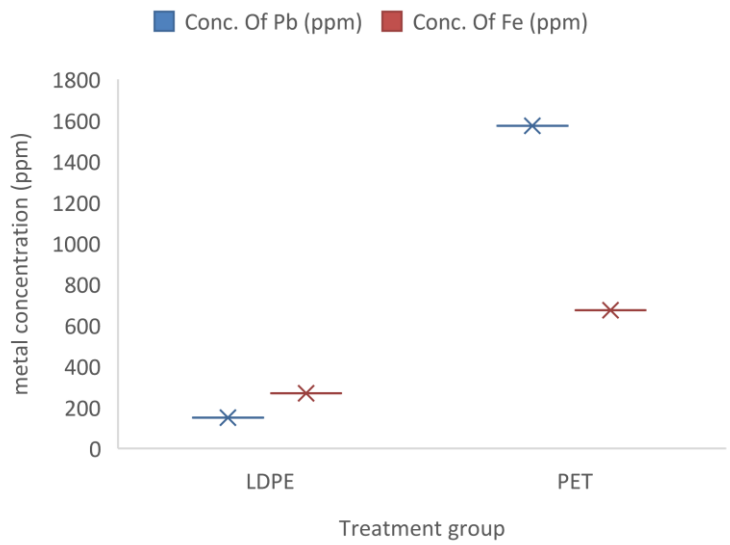

Figure 3.7: concentration of metals Pb and Fe, adsorbed on cryogenically milled PET and LDPE. X-axis represents the type of microplastic, y-axis represents the concentration of metal I n ppm, the legend shows the colour key for the type of metal (blue for Pb and red for Fe)

3.3.3. RNA expression analysis

The analysis of differentially expressed genes indicates that the differential expression of genes in some of the treatment groups was mainly related to their exposure to microplastics and their combination with metals (Pb^{2+} and Fe^{2+}). Following table 3.1 provides a list of treatment groups that showed significantly altered expression of genes in selected pathways. For the rest of treatment groups, the differential gene expression was non-significant (p-value > 0.05) and therefore are not mentioned in the list.

Table 3.1: list of treatment groups exhibiting significant differential RNA expression in three selected pathways; oxidative stress, development and reproduction. ES=Enrichment score, NES=Normalised enrichment score , p-value indicates the significance level.

GENE PATHWAY	TREATMENT GROUP	ES	NES	P-VALUE
OXIDATIVE STRESS	***Upregulated***			
	LDPE (G1)	0.61293	1.60948	0.01158
	PET+Pb (G7)	0.6129	1.88666	0.000212
	LDPE+Pb (G5)	0.49480	1.60133	1.08E-02
	PET+Fe (G8)	0.59532	1.93623	0.000358
	LDPE+Fe (G6)	0.52524	1.83802	0.001
	Downregulated			
	-	-	-	-
DEVELOPMENT	***Upregulated***			
	LDPE+Pb (G5)	0.36625	1.56103	4.21E-05
	Downregulated			
	PET+Pb (G7)	-0.2514	-1.33206	0.00031
	PET+Fe (G8)	-0.2362	-1.22493	0.01760
REPRODUCTION	***Upregulated***			
	-	-	-	-
	Downregulated			
	LDPE (G1)	-0.3683	-1.4676	0.02051

Table 3.2: list of individual genes showing significant differential RNA expression in three selected pathways; oxidative stress, development and reproduction. The G values indicates the treatment groups, +++ = strongly upregulated, ++ moderate upregulation, + = slightly upregulated, --- = strongly downregulated, -- = moderate downregulated, - = slightly downregulated.

Gene Pathway	Gene ID	Protein name	Expression
Oxidative stress	LOC124195931 (G5+++, G6+++, G7+, G8++)	vitellogenin-like	Upregulated
	LOC124195932 (G5 +++, G6+++, G7+++, G8+++)		
	LOC124207287 (G5++,G6+, G7+, G8+)	apolipoprotein D-like	
	LOC124209736 (G5++, G6++, G7++, G8++)		
	LOC124207287 (G5+,G6++, G7+, G8+)		
	LOC124202343 (G6++, G8++, G5+, G8+)LOC124200684 (G6++, G8++, G5+, G8+)LOC124196239 (G6+, G8+)	superoxide dismutase	
	LOC124209737 (G6++, G5+, G8+)	lazarillo protein-like	
	LOC124208048 (G7+, G8+)	glutaredoxin-2, mitochondrial-like	
	LOC124210004 (G7-, G5--, G6--, G8--)	mitochondrial uncoupling protein 2-like	Downregulated
Development	LOC124208163 (G5+++, G6+++, G7+, G8+)	smoothened homolog, an oncogene	Upregulation
	LOC124197298 (G5+, G7+, G8+)	neurotrophin 1-like	
	LOC124194580 (G7+++, G8+++)	uncharacterized	
	LOC124198663 (G6++, G8++, G5+, G7+)	uncharacterized	
	LOC124195424 (G5++, G6++, G7+, G8+)	GATA zinc finger domain-containing protein 10-like	
	LOC124204013 (G5++, G6++, G7+, G8+)	protein maelstrom homolog	
	LOC124194410 (G5++, G6++, G7+, G8+)	protein dimmed-like	
Reproduction	LOC124204013 (G5++, G6++, G7+, G8+)	protein maelstrom homolog	Upregulation
	LOC124190346 (G6++, G8++, G5+, G7+)	cyclin N-terminal domain-containing protein 1-like	
	LOC124202272 (G7---, G5-, G8-)	meiosis-specific with OB domain-containing protein-like	Downregulation
	LOC124193874 (G8----, G5-, G6-, G7-)	condensin complex subunit 1-like	
	LOC124192696 (G8-, G7-)	Uncharacterized	
	LOC124191926 (G6-, G7-, G8-)	doublesex- and mab-3-related transcription factor A2-like	

The data presented in the table indicates that the impact of microplastics or metals alone on gene expression was not very significant. However, when microplastics and metals interacted, there was a notable effect on gene expression at significant levels.

3.3.3.1. Oxidative stress:

The pathway most greatly impacted among the three studied pathways was found to be oxidative stress. In each of the treatment groups, it was observed that genes associated with oxidative stress were upregulated, as illustrated in figure 3.7. This observation indicates that the daphnia, when exposed to LDPE and PET microplastics containing Pb and Fe ions on their surface, experienced more oxidative stress. As a result, an upregulated expression of genes involved in defending against oxidative stress was detected.

The treatment groups with most significantly enriched genes in the upregulated and downregulated gene sets according to the normalized enrichment score are listed in Table 3.2.

Figure 3.8 shows the four most significant plots in the upregulated and downregulated gene sets.

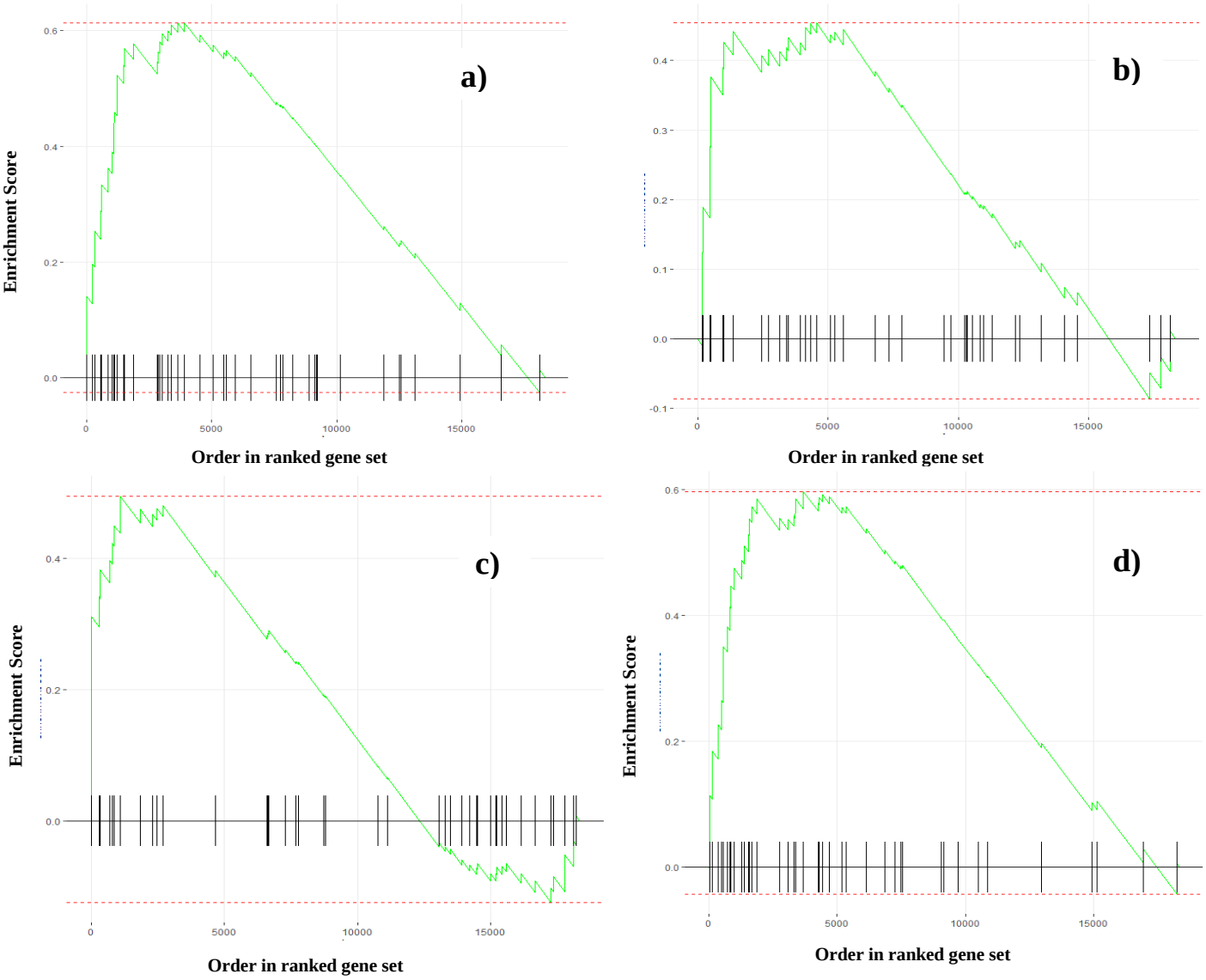

Figure 3.8: gene enrichment plots for significantly differentially expressed genes in oxidative stress pathway in treatment group a) PET+Pb b) LDPE+Pb c)PET+Fe d) LDPE+Fe. X-axis represents the order of genes in the ranked gene set, y-axis represent the enrichment score.

The green line in above plots is determined by the enrichment scores (y-axis) for the genes in the analyzed gene set, with a strong peak (either negative or positive) being indicative of non-randomly high or low ranked expression in the gene set. The x axis is the gene rank amongst all of the above-threshold

genes in the genome.

The vertical "bars" in the barcode are the positions of the gene set genes in the ranking. A negative peak gives a negative normalised enrichment score (NES) and is indicative of relative downregulation of the gene set, a positive peak gives a positive NES and is indicative of a relative upregulation.

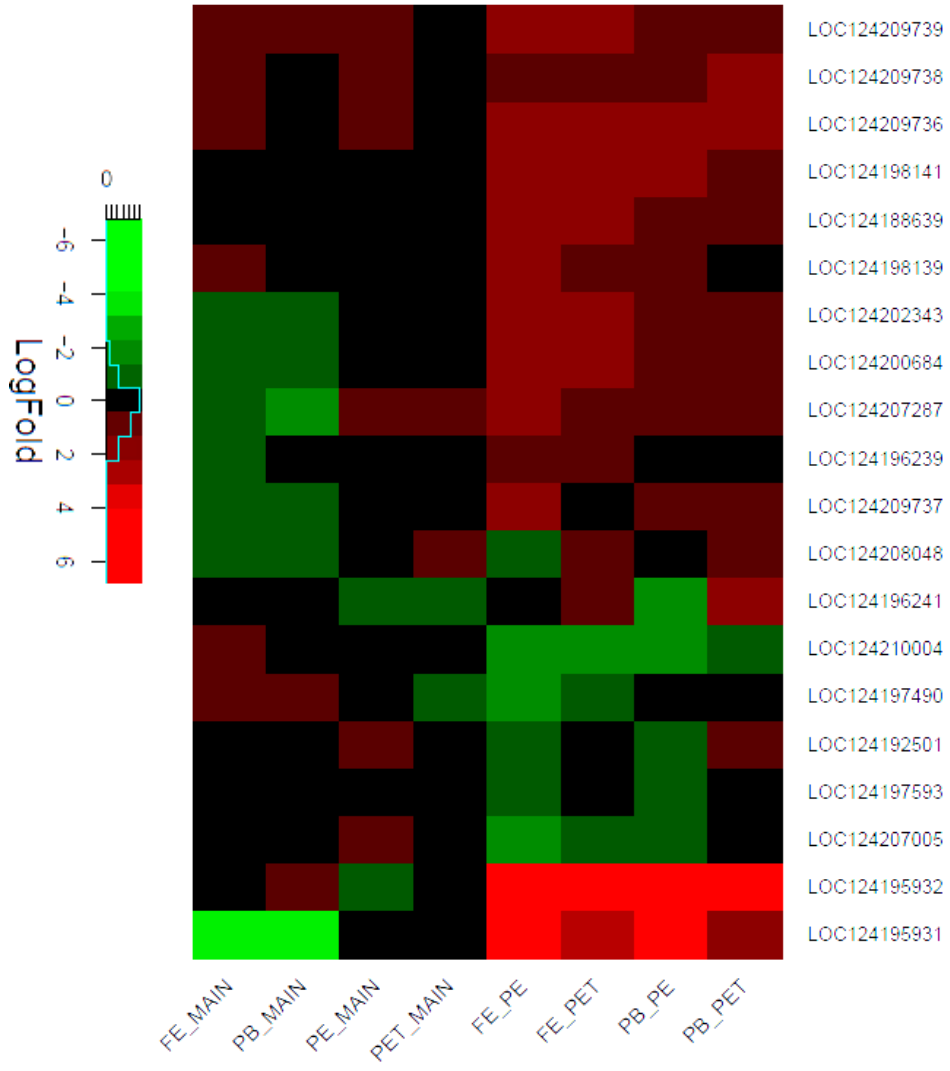

Figure 3.9: A heatmap that gives visual representation of differentially expressed genes in oxidative stress pathway for all treatment groups with log2 fold change of ≥2 and p value ≤ 0.05. the legend shows logfold value with colour indicator, red colour with positive logfold value represents upregulation and the green colour with negative logfold value indicates downregulation. Black colour, close to zero value indicates least differential expression. x-axis represents the treatment groups, y-axis represents the individual genes.

The heatmap (figure 3.9) visually represents the individual genes which showed differential expression in oxidative stress pathway for all treatment groups. The heatmap effectively distinguished between upregulated genes (with a log2 fold change of ≥ 2 and a significance level of $P < 0.05$), represented by a red colour, and downregulated genes (with a log2 fold change of ≤ -2 and $P < 0.05$), represented by green colour. According to this, out of total 54 genes related to oxidative stress pathway in *daphnia,* 20 genes were differently expressed (upregulated). Among these, the expression of LOC124195931 (vitellogenin-like) and LOC124195932 (vitellogenin-3-like) was strongly upregulated in most of the treatment groups (Table 4.) On the other hand, LOC124210004 (mitochondrial uncoupling protein 2-like (UCP) was found to be the only protein strongly downregulated in the four treatment groups (PET+Pb, PET+Fe, LDPE+Pb and LDPE+Fe). Other genes showing over expression includes LOC124207287, LOC124209736, LOC124207287 (apolipoprotein D-like), LOC124202343, LOC124200684, LOC124196239 (superoxide dismutase) LOC124209737 (lazarillo protein-like,a homologue of Apolipoprotein D), LOC124208048 (glutaredoxin-2, mitochondrial-like).

3.3.3.2. Development pathway:

The enrichment analysis indicates the significant effect of only three exposure groups (PET+Pb, PET+Fe, LDPE+Pb) on the development of *Daphnia pulex*. Figure 3.10 shows the three most significant gene sequence enrichment analysis (GSEA) plots in the upregulated and downregulated gene sets for

treatment groups PET+Pb, PET+Fe and LDPE+Pb. A total of 379 genes were linked to the development of daphnia, with 20 genes showing significant differences in expression (logfold2 fold change applied on data set) across the three treatment groups. The expression patterns of genes were found to be consistent in the PET+Pb and PET+Fe groups, with some genes being upregulated and others downregulated (figure 3.10 a, 3.10 b). In contrast, the LDPE+Fe treatment group exhibited only upregulation of genes associated with development (figure 3.10 c).

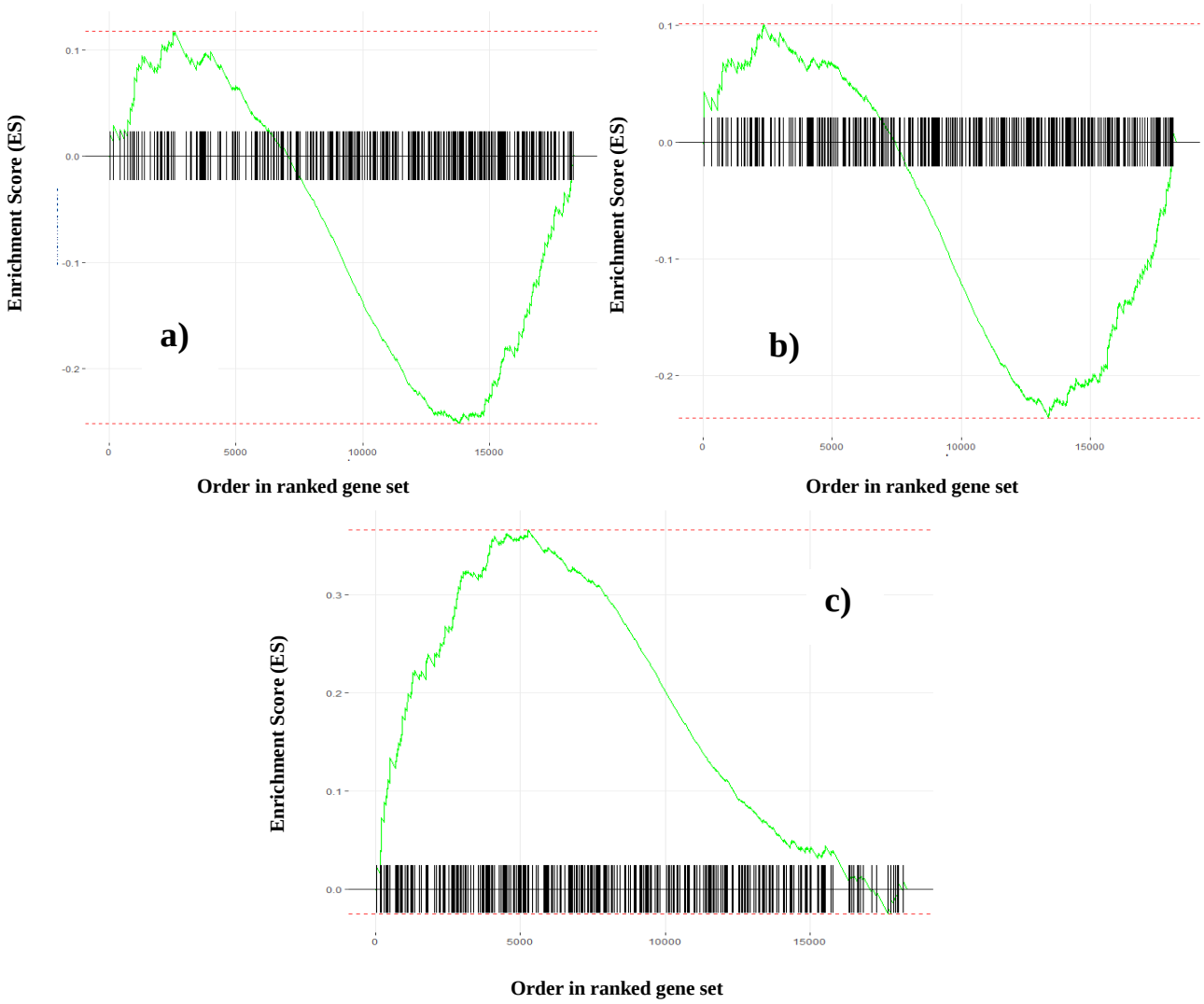

Figure 3.10: gene enrichment plots for significantly differentially expressed genes in development pathway in treatment group a) PET+Pb b)PET+Fe c) LDPE+Pb. X-axis represents the order of genes in the ranked gene set, y-axis represent the enrichment score.

The green line in the GSEA plots (figure 3.10) is determined by the enrichment scores (y-axis) for the genes in the analysed gene set, with a strong peak (either negative or positive) being indicative of non-randomly high or low ranked expression in the gene set genes. The x axis is the gene rank amongst all of the above-threshold genes in the genome.

The visual representation of individual genes exhibiting differential expression in all treatment groups is shown in the heatmap (figure 3.11). The heatmap effectively differentiated upregulated genes, depicted in red, with a log2 fold change of ≥ 2 and a significance level of P < 0.05, from downregulated genes, represented by the color green, with a log2 fold change of ≤ -2 and P < 0.05

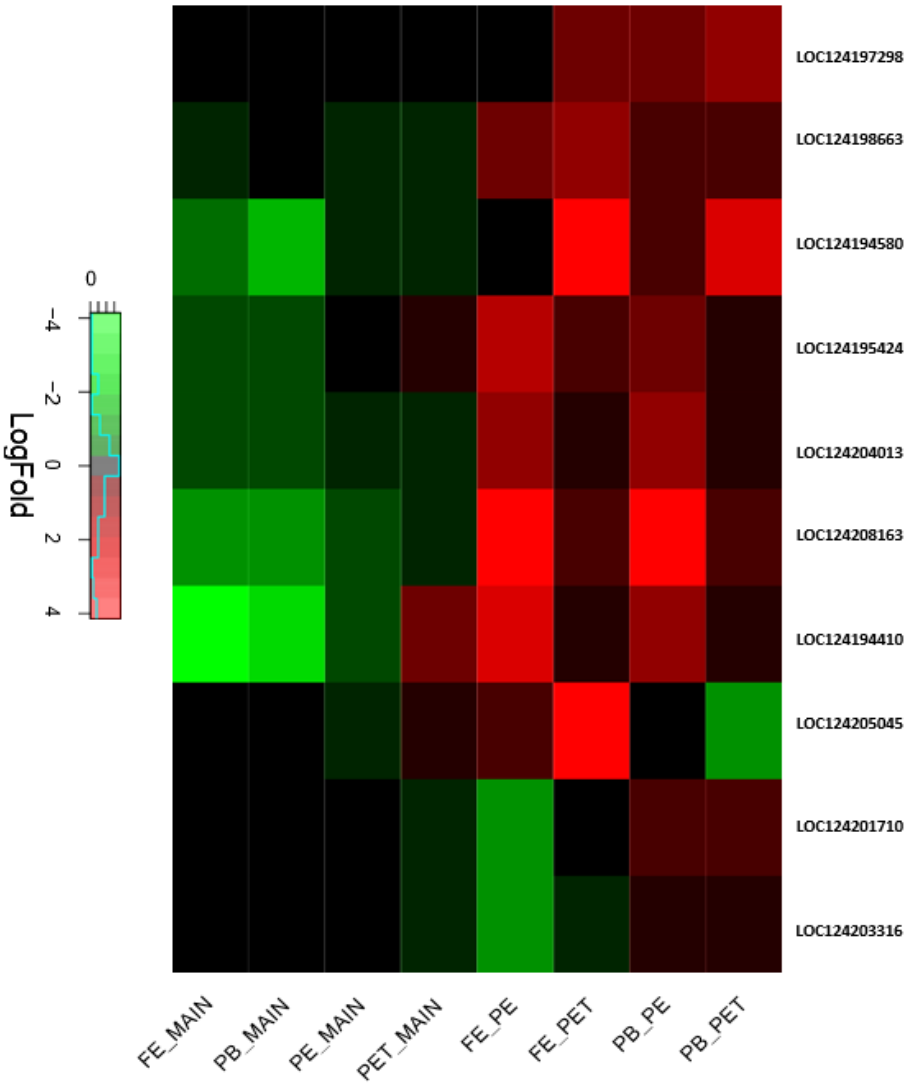

Figure 3.11: A heatmap that gives visual representation of differentially expressed genes in development pathway for all treatment groups with log2 fold change of ≥2 and p value ≤ 0.05. the legend shows logfold value with colour indicator, red colour with positive logfold value represents upregulation and the green colour with negative logfold value indicates downregulation. Black colour, close to zero value indicates least differential expression. x-axis represents the treatment groups; y-axis represents the individual genes.

Figure 3.11 illustrates relatively low expression of LOC124197298 (neurotrophin 1-like), uncharacterized LOC124198663, LOC124194580, relatively low expression of LOC124195424 (GATA zinc finger domain-containing protein 10-like), LOC124204013 (protein maelstrom homolog), high expression of LOC124208163 (smoothened homolog, an oncogene), low expression of LOC124194410 (protein dimmed-like, Loss of DIMM is associated with deficits in display of neuropeptides and neuropeptide-associated enzymes).

3.3.3.3. Reproduction

Among the three pathways selected to assess the differential gene expression in response to PET and LDPE exposure, reproduction was observed to be least significanlty affected pathway. Out of 108 genes associated with the reproduction pathway, only 14 genes showed differential expressions. From these 14 genes, expression of LOC124202272 (meiosis-specific with OB domain-containing protein-like), LOC124193874 (condensin complex subunit 1-like), LOC124192696 (uncharacterized), LOC124191926 (doublesex- and mab-3-related transcription factor A2-like) was consistently lower in the four treatment groups PET+Pb, PeT+Fe, LDPE+Pb and LDPE+Fe. On the other hand, LOC124204013 (protein maelstrom homolog), LOC124190346 cyclin N-terminal domain-containing protein 1-like showed slightly upregulation,

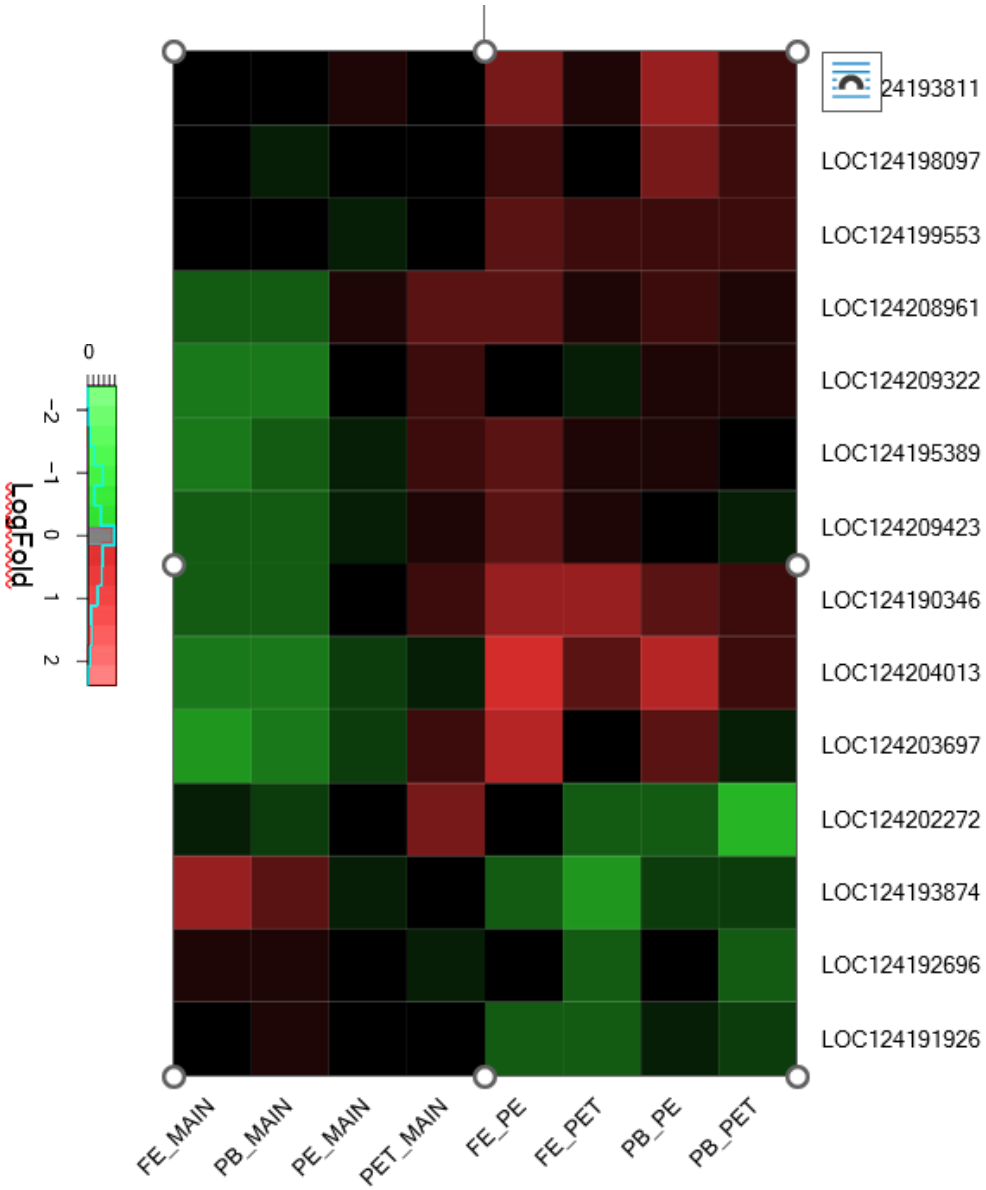

Figure 3.12: A heatmap that gives visual representation of differentially expressed genes in reproduction pathway for all treatment groups with log2 fold change of ≥2 and p value ≤ 0.05. the legend shows logfold value with colour indicator, red colour with positive logfold value represents upregulation and the green colour with negative logfold value indicates downregulation. Black colour, close to zero value indicates least differential expression. x-axis represents the treatment groups; y-axis represents the individual genes.

3.3.4. Bioassay

3.3.4.1. Body Length

The results indicated that variation in daphnia body length under all exposure groups was not statistically significant for the given period. The longest body length observed was for the Daphnia under PET exposure at day 6, with an increase of approximately 0.6mm in seven days. For all the remaining treatment groups the change in body length over a seven-day period was approximately 0.34 mm on average. Figure 3.13 illustrates the trend of body length observed in all treatment groups for a seven-day period.

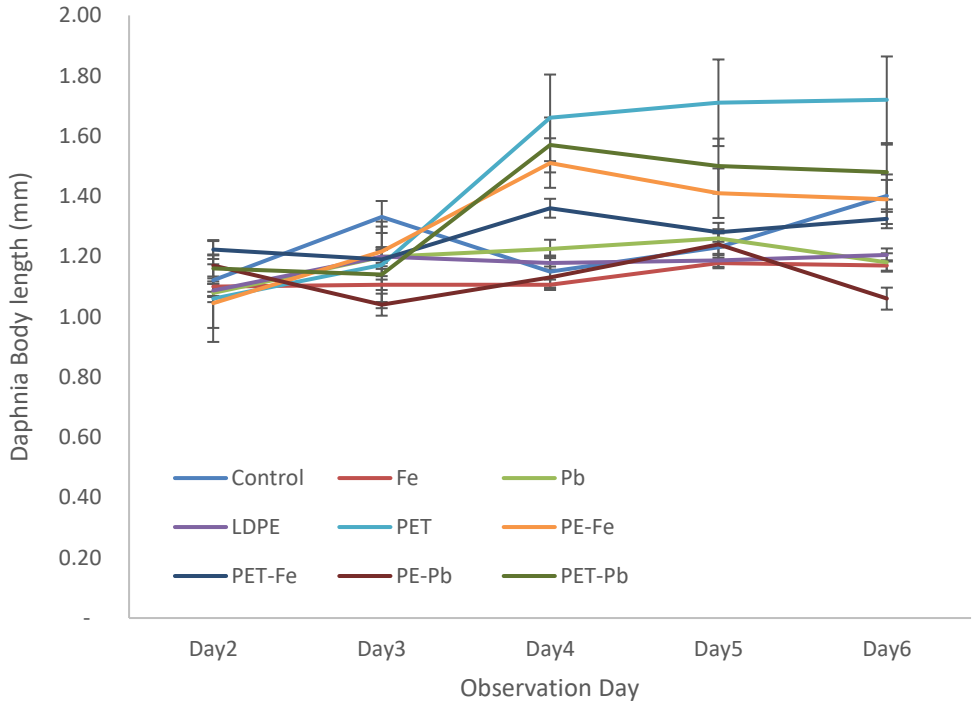

Figure 3.13 Daphnia Body length plotted against the observation day for each of the treatment group. x-axis represents the observation day, y-axis represents the body length recorded in mm, the legend shows colour code for each exposure group represented in the plot as differently coloured line.

3.3.4.2. Body Width

The statistical analysis revealed that, like body length, there was no significant difference in body width among any of the treatment groups during the specified time period. Among the treatment groups, the exposure group PET exhibited the highest increase in body width, with a change of approximately 0.31 mm over a span of seven days. In contrast, the remaining treatment groups showed a change of approximately 0.23 mm in body width over the same duration. Figure 3.14 depicts the observed trend in body length across all treatment groups over a span of seven days.

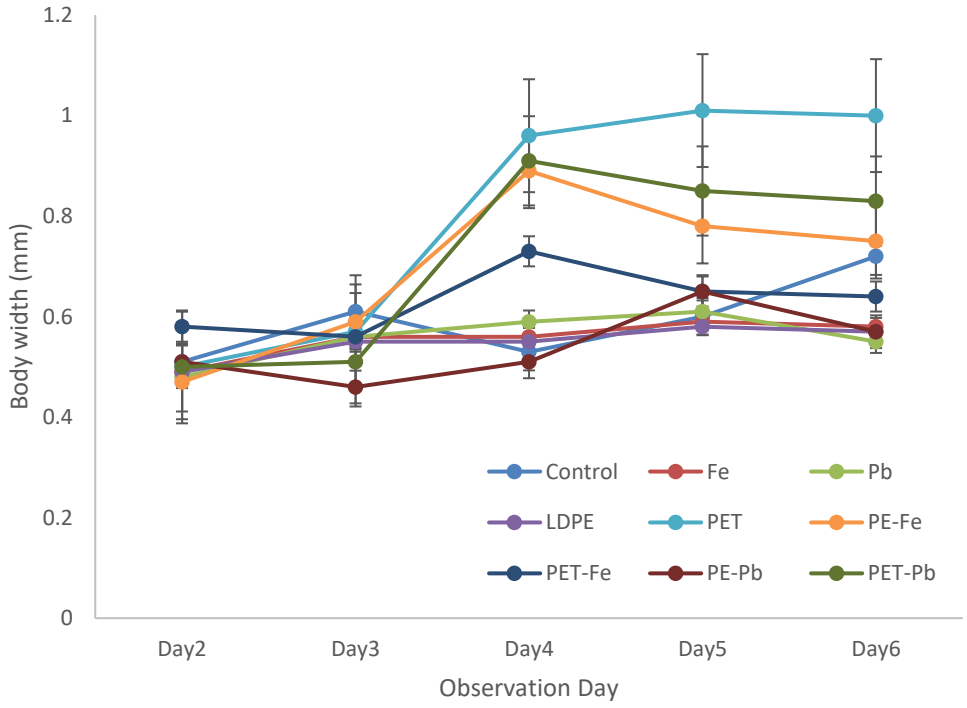

Figure 3.14 Daphnia Body width plotted against the observation day for each of the treatment group. x-axis represents the observation day, y-axis represents the body width recorded in mm, the legend shows colour code for each exposure group represented in the plot as differently coloured line.

3.3.4.3. Survival

The most notable effect of PET and LDPE exposure was on the survival of daphnia in some of the treatment groups. Where the treatment groups PET, LDPE, Fe and Pb alongwith the control showed no mortality till day seven, a significantly high mortality was observed for treatment groups of microplastics in combination with the metals (PET+Pb, PET+Fe, LDPE+FE, LDPE+Pb), with highest mortality of 100% on day five under PET+Pb exposure. PET+Fe exposure group also showed 100% mortality at day 6 (Figure 3.15).

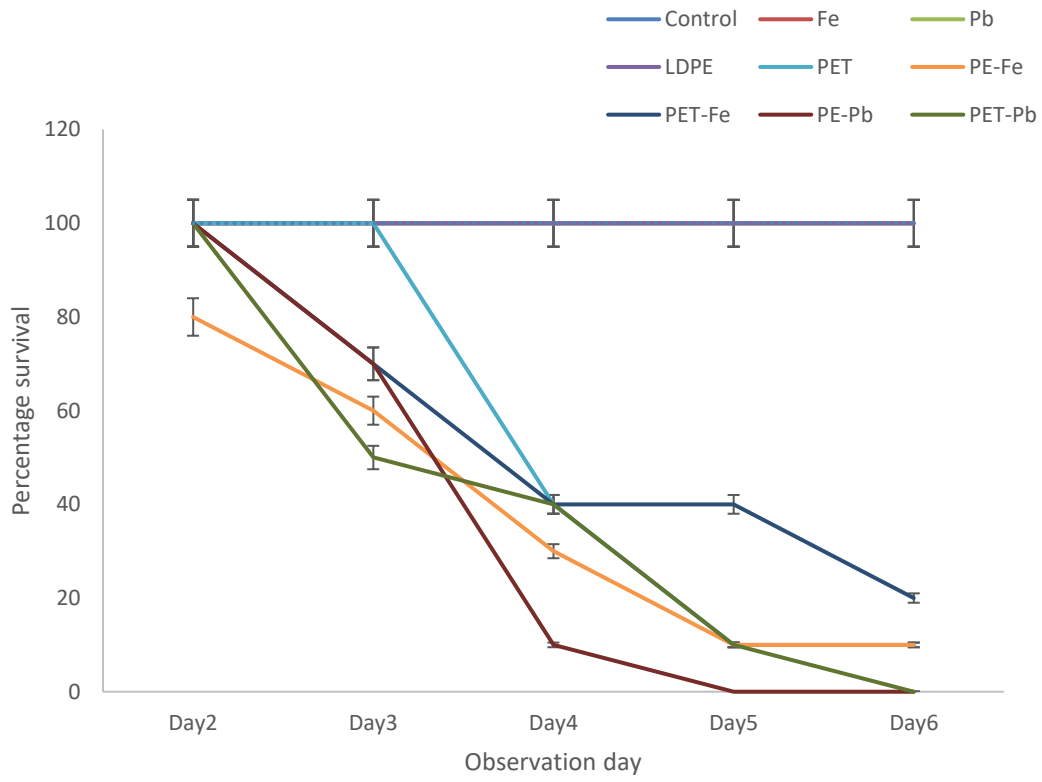

Figure 3.15 Daphnia survival rate plotted against the observation day for each of the treatment group. x-axis represents the observation day, y-axis represents the percentage survival recorded, the legend shows colour code for each exposure group represented in the plot as differently coloured line.

3.3.4.4. Heart Rate

The heart rate of individuals in all treatment groups showed a remarkable degree of variability over the course of seven days. Unlike the control group, where the heart rate remained relatively stable, the treatment groups experienced continuous fluctuations in their heart rate measurements (3.16). This significant variation in heart rate across all treatment groups suggests that the exposure groups being tested had a notable impact on daphnia's cardiac activity.

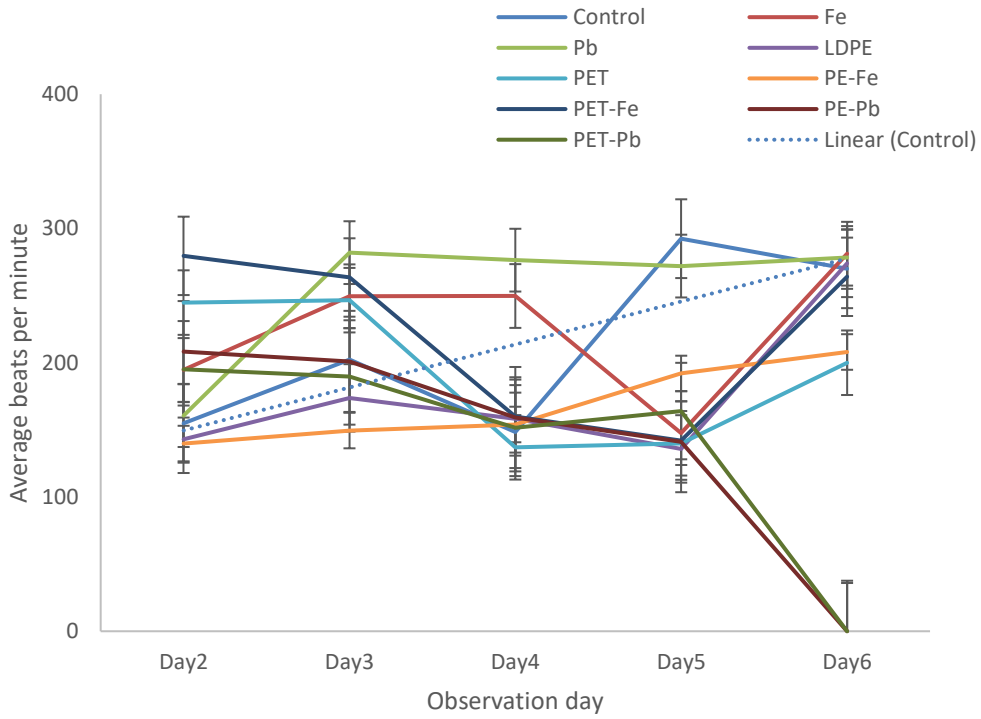

Figure 3.16 Daphnia heart rate plotted against the observation day for each of the treatment group. x-axis represents the observation day, y-axis represents the heart rate recorded as average of beats per minute, the legend shows colour code for each exposure group represented in the plot as differently coloured line.

The estimated marginal means of heart rate are plotted in figure 3.17.

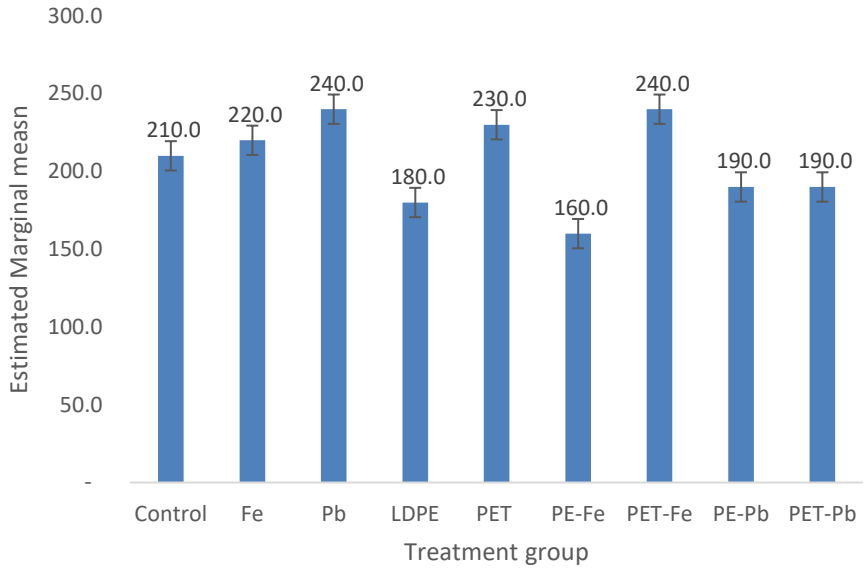

Figure 3.17 Estimated marginal means of Daphnia heart rate plotted against the treatment group. x-axis represents the treatment group, y-axis represents the estimated marginal means of heart rate\\\

3.4. Discussion

Due to the high prevalence of microplastics in freshwater environments, there is currently significant research interest in assessing their toxicological effects on model organisms. In this study, the potential toxicity of LDPE and PET, both individually and in combination with the metals Fe and Pb, was evaluated. Since these metals are found in the same environment and interact with microplastics, it is crucial to analyze their co-exposure effects on model organisms (Jeong et al., 2022). The study concluded a positive adsorption behaviour of studied metals Fe and Pb by both microplastic types (LDPE and PET). PET microplastics exhibited a greater inclination for adsorption of studied metals in contrast to LDPE. The maximum adsorption capacity was documented for Pb^{2+} on PET microplastic (figure 3.3). These findings are in accordance with Godoy et al., 2019, who concluded the highest adsorption capacity of PET for Pb^{2+} as compared to other microplastic types studied. Furthermore, the significance of PET and PE in absorbing higher amounts of Pb has also been reported by Rochman et al., 2014. However, no literature was found to compare the adsorption capacity of these microplastic type for Fe^{2+} which was investigated in this study. Following the adsorption analysis, the subsequent aim of the research was to evaluate the individual and combined toxicological impact of PET and LDPE with Pb^{2+} and Fe^{2+} on *Daphnia pule*x. To achieve this, multiple physiological measurements were recorded in controlled bioassay and molecular expression analysis of *Daphnia pulex* was

performed in response to various exposure groups. The findings indicated that while the single exposure to microplastics (at a concentration of 1 mg/l and size 10-100 µm) exhibited moderate toxicity in the 7-day exposure test, the combined exposure demonstrated relatively greater toxicity. There was no noticeable impact on body length and width in any of the exposure groups (figure 3.13, 3.14). However, the heart rate and survival did exhibit a significant effect, with the combined exposure having the highest impact (figure 3.15, 3.16). These findings differ to that of Ziajahromi et al.,2017 who reported a significant decrease in daphnia body length after exposing them to PE microplastics. This difference could be attributed to higher concentrations of PE (2mg/l) used in their experiment. However, there are some studies that report an increase in body length and width of daphnia under microplastic exposure could be taken as defensive response (Rabus and Laforsch 2021). Hanees K, 2017 also reported an inconsistent and small change in daphnia body measurement in response to PS microplastics. Hence contradictory information exists on altered morphological behaviour in response to microplastic exposure. It is likely that microplastics exposure has a range of consequences, one being physical blocking of feeding (as observed at higher exposure levels in these experiments occurring as well as sub-lethal effects, and possibly co-occurring.

Reproduction was observed to be affected by all of the treatment groups in the studied period of time. In seven days period only one brood was released

and none of the organisms produced a second brood. This agreed with Gersan An, 2024 who reported a reduced reproductive activity in response to PET microplastics. However, in this study low statistical significance was reported (p=0.005) and further studies with larger numbers, longer exposure times and varying exposure levels would be necessary.

Effects on daphnia mortality were observed to have the greatest statistical significance in terms of the biological response of microplastic exposure in this study which is in agreement with many previous studied that have reported an increase in rate of mortality in response to different microplastic types ((Jemec et al., 2016; Rehse et al., 2016; Frydkjær et al., 2017; Song et al., 2021). Naetal 2023 suggested that the chronic toxicity of microplastics induce oxidative stress that leads to high mortality rate as supported by the molecular analysis showing up-regulation of antioxidants (table 3.1, 3.2, fig 3.8, 3.9). The findings are also consistent with Lei et al 2018 who reported reduced survival rate in response to PE microplastics.

The results from bioassay experiment clearly demonstrated the sub-lethal effects of both microplastics types in combination with the studied metals, hence RNA expression analysis was performed to validate the impact at molecular level. The results of RNA expression analysis in this study strongly corelates with the physiological responses observed. Three biochemical pathways namely Oxidative stress, Development and Reproduction were studied to assess the impact of PET and LDPE single and combined exposure.

Oxidative stress has widely been explored as key indicator of microplastic induced toxicity (Wang et al., 2021; Vlahogianni et al., 2007). As defined by Sieus (2020). Oxidative Stress is an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage. Increased energy demand and oxygen requirements during stress lead to imbalance of reactive oxygen species and expression of antioxidants indicates the organism is trying to restore homeostasis. The results from this study demonstrated that genes related to oxidative stress defence were significantly upregulated (fig 4.8) with most differential expression observed in this pathway as compared to the other two pathways studied. Expression of superoxide dismutase was in particular very high. Superoxide dismutase is one of the widely studied oxidative stress biomarker (Prokic et al., 2019). Under oxidative stress conditions, superoxide dismutase (SOD) acts as a first line of defense, catalyzing the conversion of reactive oxygen species to hydrogen peroxide, which is then converted to water and oxygen by catalase (CAT) or peroxidase (Esterhuizen-Londt et al., 2011).

The findings agree with those obtained by Wang et al., 2021 and Vlahogianni et al., 2017 who reported an upregulated expression of SOD under PS microplastic exposure. Catalase (CAT) and Glutathaione (GTx) are also common indicators used to study oxidative stress responses in daphnia (Wang et al., 2021; Vlahogianni et al., 2007). However, our study did not find any

differential expression of these two enzymes in any of the treatment groups, however SOD was observed. The second gene that showed relatively stronger upregulated expression is LOC124195931 (vitellogenin-like) and LOC124195932 (vitellogenin-3-like). Vitellogenins are major egg-yolk protein prceursors expressed in most of the oviparous animals and serve as a source of energy supply for embryo development ((Hannas et al., 2011; Subramoniam, 2010). However, expression of this proteins has also been related to stress conditions such as thermal stress (Matozzo et al., 2008; Schwerin et al., 2009), estrogenic chemicals (Hannas et al., 2011), metallic nanoparticles induced oxidative stress (Rainville et al., 2014). The role of vitellogenins in embryonic development has been well studied in daphnia but the mechanism adapted by these proteins in stress response is still not investigated. LOC124210004 (mitochondrial uncoupling protein 2-like) strongly downregulated in the four treatment groups (PET+Pb, PET+Fe, LDPE+Pb and LDPE+Fe). UCPs are the mitochondrial proteins that provides a strong defence against mitochondrial oxidative stress in all eukaryotic organisms by increasing membrane conductance for protons and lowering protonic backpressure on respiratory chain (Mendez-Romero et al.,2020). A study by Mendez-Romero (2020) first time characterize the increased expression of mitochondrial UCP under oxidative stress in white leg shrimp. Other genes showing over expression included LOC124207287, LOC124209736, LOC124207287 (apolipoprotein D-like), LOC124202343, LOC124200684, LOC124196239 (superoxide dismutase)

LOC124209737 (lazarillo protein-like,a homologue of Apolipoprotein D), LOC124208048 (glutaredoxin-2, mitochondrial-like). Multiple physiological role of apolipoproteins such as anti-inflammatory and antioxidant roles have been reported in various studies.(Castex et., 2010, Yuqi Mu et., 2023). Yuqi Mu (2023) investigated the expression of Apolipoproteins in soft shell turtles in response to Florofenicol induced oxidative stress.

The overexpression of SOD alongwith other oxidative stress related genes under the exposure of microplastics with metal adsorbed indicates that microplastic exposure led to oxidative stress, which ultimately impacted the physiology of the organisms involved in the bioassay conducted during this research. The confirmation by biochemical assay of antioxidant activity followed by investigation of these genes' involvement in stress regulation presents a field for future research, and these could be potential biomarkers to be recognized as novel indicators of the response to environmental stress.

This study also evaluated the expression of reproduction and development related genes and found they were downregulated in most of the treatment groups. There were only small number of genes which showed differential expression in these pathways. Low expression of neurotrophins (LOC124197298) was detected in three treatment groups (PET+Fe, LDPE+Pb and PET+Pb). Neurotrophins role in neural development has been well studied in mammals but expression of neurotrophins in crustaceans is detected only recently (Wilson et al., 2009). Low expression of neurotrophins suggests the

compromised Daphnia growth under microplastics and metals exposure rather than complete growth inhibition. However, more evidence is required to relate its role in Daphnia's embryonic development. Higher expression of LOC124208163, smoothened homolog SMO, an oncogene was detected in treatment groups LDPE with Fe and Pb compared to PET exposure groups. Contrary to this its role was strongly downregulated in the Fe single and Pb single exposure groups. The role of SMO is well studied in higher vertebrates and some invertebrates as an oncogene, associated with hedgehog signalling pathway responsible for cell growth and differentiation, but there is no literature that reports the expression of SMO in daphnia. Some of the genes exhibiting differential expression are not yet characterized, thus offering a promising field for future investigation to gain insight into stress responses. and into their functions. The results from RNA expression analysis could be further verified by qPCR analysis which will provide a strong validation of differential expressions observed in these experiments.

The findings obtained from the RNA seq analysis align with the chronic toxicity assay results in the same study, which indicated sub-lethal impact of the treatment groups on both body size and reproductive activity.

3.5. Conclusion

The study investigated the impact of cryogenically milled microplastics LDPE and PE alone and in combination with metals Fe and Pb on *Daphnia* at the physiological as well as molecular level. In the exposure assays, the microplastics were prepared in

lab using cryogenic milling which aims to produce microplastics with rough surface morphologies and broad size range to mimic microplastics present in natural environment. A chronic toxicity assay conducted at 1 mg/l exposure concentration for 7 days and concluded that this exposure showed no major effects on the *Daphnia's* body length and width. Heart rate did not show any specific pattern in the observed period, whereas the reproductive activity was observed to be negatively affected. The most obvious impact of all the treatment groups was observed on organisms survival, with the death of *Daphnia* being reported from the third day of observation (20% mortality in LDPE+Pb and 40% in LDPE+Fe). The highest mortality (100%) was observed at day5 in LDPE+Fe. The impact on each of the physiological endpoint was higher in the microplastic and metal combination exposure groups compared to single exposure groups. suggesting sublethal effects in these experimental groups. Differential RNA expression analysis was performed. The results from this analysis correlates to the bioassay experiments. In the RNA Seq analysis, three biochemical pathways were assessed namely Oxidative stress, Development and Reproduction. Oxidative stress was the most significantly affected pathway with most of the genes observed to be upregulated particularly in the microplastics and metal combined exposure groups. This suggests that these treatment group induced the oxidative stress mechanism and most of the biochemical resources are utilized to restore redox homeostasis and thus the development of the organism as well as the reproductive activity is compromised.

Based on the data presented in this study, the metal-adsorbed microplastics resulted in sub-lethal oxidative stress as an adverse outcome of exposure.

Chapter 2, section 2.3.3 shows that sampling of freshwater from the targeted river found 0.1ppm microplastics, which is below the threshold concentration used in this bioassay, however flushing of microplastics in rivers by surface run-off process can cause local increases (Horton et al., 2017**).** Thus even at levels of 0.1% (1mg/L) the combined microplastic toxicity with heavy metals can have impacts on physiology, biochemistry, growth, development and reproduction and thus affect the survival and health of freshwater invertebrate populations in natural environments. Consequently, additional research should be conducted to explore the collective toxicity of microplastics with different metal types and other categories of pollutants on freshwater organisms.

Chapter 4

Microwave assisted thermal Degradation of Cryogenically Milled PET microplastic particles and the toxicological impact of the degraded intermediates on *Daphnia pulex*.

4.1. Introduction

Due to the durable nature of plastic particles, they are considered as persistent organic pollutants in the environment. Either primary or secondary sourced, the microplastic particles readily enter wastewater treatment plants where they undergo wastewater treatment process. Wastewater treatment, also known as domestic or municipal wastewater treatment, is a process that purifies sewage by removing contaminants. The objective is to generate an effluent that can be safely released into the environment or utilized for a specific purpose, thus avoiding the pollution of water bodies due to the discharge of untreated sewage. The different pathways adapted by microplastic to enter wastewater includes (Ngo et al., 2019):(a) personal care products (such as toothpastes, facial cleansers, and body washes) related household effluent, and fibers in laundry wastewater (Browne et al., 2011; Yang et al., 2021); (b) Numerous wastewater collection systems established as a result of the unregulated release of industrial wastewater (c) storm water related dry and wet deposition, which mainly refers to the plastic debris suspended in the atmosphere; and (d) landfill leachates. In terms of microplastic concentration, the average concentration of microplastics varies from 2.5 ± 0.3 items/L to 1106 ± 651.9 items/L in the influent and 0.11 ± 0.11 items/L to 208.55 ± 77.61 items/L in the effluent of the global wastewater treatment plants which suggests that the concentration of microplastics in the influent is significantly higher than that in effluent. Despite the significant

presence observed in the influent, wastewater treatment process has been shown to unintentionally exhibit remarkable effectiveness in eliminating microplastics from the wastewater flow. Research has reported removal efficiencies ranging from 57% to 99% following secondary treatment processes (Carr et al., 2016; Gies et al., 2018; Liu et al., 2019). The notable removal efficiency indicates that the majority of microplastics are effectively captured and retained in the sludge during wastewater treatment (Sun et al., 2019). Sewage sludge, in turn, is the solid residue generated during the wastewater treatment process. Microplastics in the sludge are dispersed in the environment through different routes such as agricultural land or underground water and thus become part of environment again (Aubain et al., 2002).

4.1.1. Wastewater treatment process

Our knowledge of microplastic degradation in water mainly comes from Wastewater processing. When microplastics passes through the three stages of wastewater treatment process; primary, secondary, and tertiary plants, it undergoes various biotic and abiotic weathering processes. These processes include mechanical abrasion, chemical oxidation, and biodegradation, which are influenced by factors such as sands, bacteria, fungi, chlorine, UV, or ozone. As a result of these processes, microplastics experiences changes in its physicochemical properties like surface morphology, crystallinity, and hydrophobicity and further downsizing into abundant secondary

microplastics, indicating that WWTPs are crucial producers for microplastics in aquatic environments (Wu *et al*., 2022).

The three stages of wastewater treatment process during which microplastics undergo degradation are discussed below;

4.1.1.1. Primary treatment stage

After the preliminary screening for larger plastic pollutants from waste water, the retained microplastics are transported to the primary treatment plant , where they are prone to endure varied shear stress forces caused by riverbeds, pipes, and drains through mixing or pumping (Enfrin *et al*., 2020). It leads to mechanical degradation of microplastics with altered surface morphology and brittleness (Enfrin et al., 2020; Song et al., 2017).

4.1.1.2. Secondary Treatment stage

After entering the secondary part of the wastewater treatment process, microplastics certainly face reactive sewage sludge, which includes various types of plastic-degrading microbes (i.e., *Hyphomonadaceae, Flavobacteriaceae, Rhodobacteraceae, Pseudomonas, Ideonella sakaiensis, Bacillus cereus* and *Bacillus gottheilii*) in the biodegradation reactor which have potential to degrade plastic polymers into monomers and oligomers (even into CO_2, H_2O, and CH_4) as a result of enzyme reactions (De Tender et al., 2017; Wei et al., 2019a; Wu et al., 2022; Yoshida et al., 2016). The role of microbes in degrading microplastics is thus very important and is discussed in detail in the following section.

- **Biodegradation**

Biodegradation is a very important degradation process for plastic particles. However, in the environment this degradation process is coupled with mechanical or physiochemical degradation. Though the plastic particles are not particularly susceptible to microbial attack as other degradable products (Rujnic-Sokele and Pilipovic, 2017) still they provide good environmental niche for microbial colonization by supporting growth and serving as a carbon source. Several microbes have been identified that are involved in plastic particle degradation such as bacterial pure cultures as well as, fungi and biofilms, and a significant decrease in weight of plastic particle has been observed (Devi *et al*., 2015).

A) Bacterial Mediated Biodegradation

Bacteria are notorious for their extraordinary abundance in every compartment of the environment. Besides their abundance, bacteria gained due scientific attention due to its potential ability to degrade various environmental pollutants (Bakir *et al*., 2014). In recent years, some pure bacterial strains have been reported to degrade micro plastic particles. An investigation by Auta *et al*., 2018, used two pure bacterial cultures collected from mangrove sediment and used for micro plastic particle degradation. Auta also assayed the ability of two isolates, *Bacillus cereus* and *Bacillus gottheilii*, to degrade different types of MPs. The results interestingly revealed that microbial activity causes several pore and irregularities on the surface of

treated micro plastic particles. This concludes that the pure bacterial cultures do have potential to adhere, colonize and damage microplastic particles. However, studies suggest that the weight loss of microplastic particles as a result of bacterial degradation is very low such as 0-15% and hence plastic particles could be said as poorly biodegradable particles (Yuan *et al*., 2020). On the contrary to this, there are some studies which suggest that pure bacterial cultures produce some toxic compounds that inhibit its growth (Dobretsov *et al*., 2013). These findings are further supported by evidence that encourages use of combination of bacterial strain for degradation of microplastics. The combination of bacterial strains, bacterial consortia, serves better for degradation purposes because it creates stable bacterial community by inhibiting the toxicity of compounds released by individual strains. In this scenario, the toxic chemicals released by one strain could be used as substrate for other bacteria thus nullifying its effect on bacterial community and increasing biodegradation potential (Singh and Wahid, 2015). Moreover, a study by Tsiota *et al*. 2018 demonstrated that two different consortia show different potential for degradation of plastic particles. However, the degradation of microplastic polymers through bacterial consortia is a complex process and so far, the respective studies have shown potential only under controlled conditions so extensive research is required to develop an efficient bacterial consortium in open environment to have maximum degradation potential consequently reducing microplastic

pollution.

B) Fungi Mediated Degradation

Like bacteria, fungi have also been of great interest to the scientific community because of its wide distribution as well as beneficial environmental potential. Recent studies have explored that fungi can adhere to and interact with microplastic particles (Mitik-Dineva *et al*., 2009). With microplastic particles, fungi can promote making chemical bonds such as carboxyl, carbonyl or ester bonds that results in decrease of hydrophobicity of microplastic particle. Moreover, fungi may encourage the transformation and circulation of different substances (Chen et al., 2016). In one such study by Sangeetha Devi (Devi *et al*., 2015) two fungi species were investigated for their MP degradation potential. First species investigated was *Aspergillus tubingensis* VRKPT1 collected from marine coastal area and was tested on high density polyethylene microplastic particles with 30 days exposure time. Results showed that there was a significant decrease in weight of plastic particle and used it as carbon source. Another species investigated was *Aspergillus flavus* VRKPT2 which demonstrated similar results on high density polyethylene particles (Devi *et al*., 2015). An investigation by Russell *et al*., 2011 demonstrated that fungi have potential to degrade different microplastics such as polyurethane (PUR). The research identified a serine hydrolase enzyme which was responsible for the degradation of polyurethane microplastic particles, suggesting that the enzymes secreted by

this fungus have potential for microplastic biodegradation. At present, many high-density polyethylene (HDPE)- degrading fungal strains have been discovered from polyethylene waste discarded in marine coastal areas and assessed under *in vitro* conditions. These results displayed the excellent proficiency of these fungal strains to degrade microplastics under *in vitro* conditions. Thus, future studies could involve the use of metagenomic mining techniques to increase the discovery rate of fungal-mediated MP degradation (Paco *et al*., 2017).

4.1.1.3. Tertiary treatment stage

In contrast with the physical damage to the microplastics in the primary treatment stage and biodegradation in the secondary treatment stage, chemical disinfection (UV-associated photooxidation, ozone oxidation, and chlorination) in WWTP tertiary treatment stages (the final step before microplastics emission into natural waters through effluent) can also cause poorly reversible damage to microplastics characterizations. Some of the important degradation methods of tertiary treatment process are elaborated below;

- **Photodegradation (UV catalyzed Oxidation)**

Degradation of microplastic polymers by long-term exposure to sunlight, enriched by UV rays is regarded as a key degradation process occurring in nature. UV light being the major influencing factor acts on polymer molecules and induces morphological changes to polymer surface such as flakes and cracking (Cai et al., 2018), as well as chemical changes by producing environmentally free radicals, addition of oxygen, subtraction of hydrogen, and scission or cross-linking of chemical chains (Zhu et al.,

2019). Owing to the significance of this uncontrollable natural process, it is very important to investigate the relation between the aging process of microplastics and the extent to which degradation could occur in specific period of time. Until now, very few studies have paid attention to the properties of photodegraded intermediates of microplastics and assessing the extent to which degradation occur in environment. In this regard, a recent study conducted by Zhu et al. (2020) investigated the process of aging in polystyrene microplastic particles under model aquatic environment. The study used stimulated sunlight with wavelengths ranging between 295 to 2500 nm, for a period of 150 days. The presence of free radicals such as singlet oxygen ($^1O_2$), •OH, H_2O_2 and superoxide radical ($O_2^{\bullet-}$) in the suspension of polystyrene microplastics indicated that photodegradation does occur in given experimental conditions. However, the study could not evaluate the degree of aging as well as the degradation intermediate products produced during the photodegradation process.

Concluding, the process of UV induced photodegradation is found to be extremely slow particularly in aquatic environments (Du et al 2021). Moreover, long-term exposure to simulated light irradiation on a larger scale result in much more energy consumption as well as sunlight pollution. Use of additional factors to facilitate the photodegradation process such as oxidants (as done in wastewater treatment) can further produce secondary pollution due to leaching of iron ions and a great deal of sludge formation inhibits its practical application (Zhou et al., 2016, Duan et al., 2018).

Thus, the microplastics returning to the environment from the wastewater treatment plants have altered chemical properties and are expected to interact differentially

to the other pollutants in the environment such as heavy metals and so have different toxicological effects on water biota.

Besides the unintentional degradation of microplastics in wastewater treatment plants, attempts are made to devise specific methods for degradation of microplastics to reduce their toxicological effects and converting them into useful products such as fuel. In this regard, the potential of thermal treatment is widely explored.

4.1.2. Thermal Degradation

Degradation of plastic waste, particularly microplastics through UV induced photodegradation or microbe-mediated breakdown have certain limitations that limit its application on large scale. Thermal degradation under very controlled conditions, on the other hand, serves as a relatively better approach to break microplastic particles and convert them into smaller molecules, including gases and liquid hydrocarbons. This thermal application could be targeted to the sludge concentrated with microplastics, to avoid excessive use of resources for thermal treatment on large amount of water.

A lot of research is going on to discover effective ways to degrade long-chain plastic polymers into higher quality products at various temperature and pressure conditions. For example, Gasification is a fuel production process in which waste is heated in a vessel to produce a gas that can then be used as a fuel or as an intermediate for chemical or fuel production (Goad et al., 2017; Sarker et al., 2012). Another study conducted by Walendziewski used plastic waste (waste comprised of three types of plastics polyethylene, polystyrene, and polypropylene) as raw materials in their experiments to carry out polymer cracking at different

temperature, pressure and catalytic conditions (i) 350°C to 420°C at atmospheric pressure and ii) 3 to 5 MPa with temperature range of 380°C to 440°C). The study concluded that the application of catalyst resulted in lowering of polymers cracking temperature and density of liquid fuel. No doubt, thermal degradation is found to be a relatively effective method for plastic waste management by converting it into useful products such as fuel. However, like other methods used for plastic degradation, thermal degradation also has some limitations, the most important being the release of chemically active microplastics into the waste. When released into the environment, microplastics (MPs) have the potential to affect both marine and terrestrial ecosystems because of their tough nature. As discussed in the above section, most current wastewater treatment facilities remove microplastics from wastewater, but they do so at the expense of concentrating them in sludge.

4.1.2.1. Impact of thermal treatment on Surface Morphology and Chemical nature of PET microplastics

Although plastics are complicated and difficult to degrade, exposure to abiotic factors such as heat, light, gases, water, and mechanical factors may cause alterations in their structure and partially induce deterioration (Lucas et al., 2008; Iram et al., 2019). At the glass transition temperature, polyethylene terephthalate (PET) is sensitive to hydrolytic breakdown in water by breakage of ester bonds (Ballara and Verdu, 1989; Allen et al., 1991). Moreover, under acidic and alkaline circumstances, the rate of conversion is accelerated, resulting in the production of carboxylic acid and alcohol functional groups. Hence the process of hydrolysis in PET is autocatalyzed once the carboxylic end groups are produced (Figure 4.1) (Hosseini et al., 2007, Gewert et al., 2015). The degradation of PET MPs under thermal or acidic/alkaline conditions makes them chemically more active and susceptible to

degrading more if subjected to further digestion procedures.

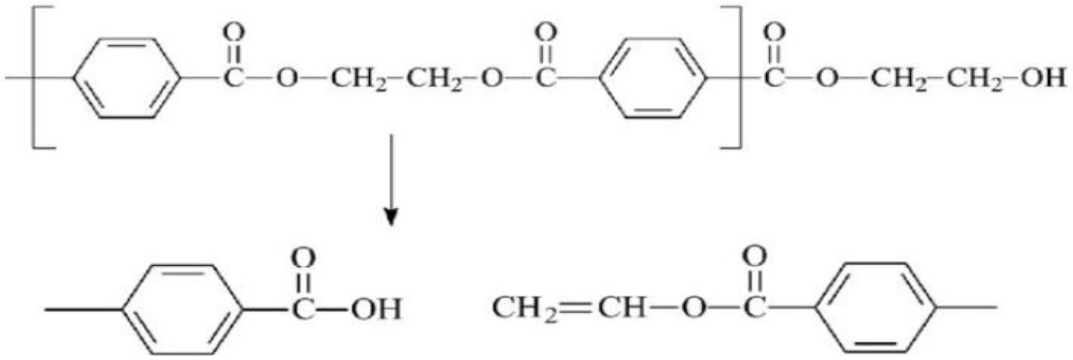

Figure 4.1. Schematic diagram for thermal degradation of PET microplastic polymer with the formation of carboxylic acid and viny ester groups

Given the significant changes in the chemistry of PET MPs after thermal treatment, it is imperative to investigate their physiochemical properties, the degree of degradation, nature of intermediate degraded products and the toxicological impact of the degraded PET MPs on living organisms. So far, no direct investigations have been made on these above-mentioned issues.

Two related research studies indirectly investigated the impact of thermal treatment in alkaline conditions as a pretreatment procedure to evaluate its impact on anaerobic digestion of sludge containing microplastics. First, Zeng et al. (2022) found that among the various sludge pretreatment processes (i.e., ultrasonic alkaline, Fenton, thermal), the thermal hydrolysis process (THP, at 70 °C for 1 h) yields the highest methane yield suggesting the alterations in physiochemical properties of studied microplastics. However, they did not monitor changes in MPs-related

properties other than the leaching of sodium dodecyl sulphate (SDS). Second, Azizi et al. (2022) discovered that THP reduces ROS generation to counterbalance the inhibition of methane synthesis brought on by high polystyrene nanoplastic levels in sludge. They did not examine the fate of nanoplastics either, even though they focused on the possibility of THP as a remediation technique to reduce the stress caused by NPs on anaerobic digestion.

The target river Mersey for the present study has a long history for being heavily polluted. Pollution on the river Mersey has been a persistent issue since the industrial revolution of the 18th century. Its existence was acknowledged as early as the mid-19th century, and it reached its peak in the mid-1960s. During this time, a combination of sewage effluent and a complex mixture of inorganic and organic chemicals from factories in the Mersey catchment and along the estuary shores caused severe contamination (NRA, 1995; Jones, 2000). Fortunately, significant improvements have been made in recent years (Jones, 2000). However, substantial amounts of organic and inorganic contaminants remain in the estuarine sediments.

Given the importance of thermal treatment for effective degradation of microplastics, the problem of heavy metal contamination and their interaction in the environment, the present study aims to investigate the potential of microwave assisted thermal treatment under 3 solvent conditions (in relevance with the pH conditions of waste treatment processes) to degrade PET microplastic particles (with size range 10um-50 um) and to study the impact of this on the chemical nature of degraded microplastics.

Based on the understanding from literature, it is hypothesized that degradation will alter the surface properties of the MPs and alter the likelihood of degraded PET MP

to adsorb heavy metals. The toxicological effects of these metals interacting microplastics after thermal treatment will be evaluated on fresh water living organism (*Daphnia*).

4.2. Materials and Methods

The degradation of cryogenically milled PET particles was analysed by suspending it in three different solvents i-e water, HCL and Acetone. The degradation was induced by a microwave-assisted thermal treatment. The cryomilled PET particles were allowed to interact with metal solutions (Pb_2SO_4 and Fe_2SO_4 solutions in milli-Q water) and the adsorption behaviour was analysed using XRF. Two-way analysis was performed to evaluate the effect of microwave-induced thermal treatment i) the chemical analysis performed to investigate the chemical nature of thermally treated PET particles using FTIR and NMR, ii) Bioassays were performed to assess the toxicological effects of thermally treated PET particles on model organism (*Daphnia pulex*).

The preparation and characterization of PET microplastics reference material was done as explained in chapter 4, section 4.2.1. Fischer Scientific FTIR instrument was used to analyze the effect of microwave assisted thermal degradation on cryomilled PET particles. A very small amount of dry sample (PET particles from each of different treatment groups) was placed on the crystal and clamp was turned down tightly to hold the sample in closest proximity to the crystal, to allow infrared light to pass through the sample.

The benchtop NMR by BRUKER was used to cross evaluate the effect of thermal

treatment on degradation of PET microplastic particles in each treatment group. The solvents separated from each treatment group were used as samples for this analysis.

4.2.1. Chemical Analysis

Treatment group 1: PET/water system:

A) 6 suspensions, each containing 10 mg of cryo-milled PET was suspended in 10 mL of distilled water and left to equilibrate overnight. After being equilibrated, the solids and liquid components of the mixture were separated via decantation. The solid component was analysed by infrared spectroscopy to check for the effects of thermal treatment on chemical nature of the PET and the liquid component by bench top NMR to know if there are any degradation products released into the solvents after being thermally treated.

B) Three of the above six suspensions of 10 mg PET in 10 mL of distilled water, equilibrated overnight was treated in the microwave at 75^{O}C for 15 min. Separation of solid and liquid phase was done as mentioned above. Solid component was analysed by IR and the liquid component by bench top NMR.

C) Approximately 5mg of the solid extracted from in A and in B was used for adsorption of heavy metals from $FeCl_3$ and $PbCl_2$ aqueous solutions. The metal loading on the solid is determined by XRF.

Treatment group 2: PET/acetone system:

A) 6 suspensions of 10 mg of cryo-milled PET suspended in 10 mL of HPLC-grade acetone were prepared and left to equilibrate overnight. The solid and liquid phase

extraction was performed via decantation method. Solid component was analysed by infrared spectroscopy and the liquid component by bench top NMR.

B) Three of the above six suspensions, equilibrated overnight, were treated in the microwave at 75 C for 15 minutes. The solid component is analysed by IR and the liquid component by bench top NMR.

C) Approximately 5 mg of the solid extracted from A and in B was used for adsorption of heavy metals from $FeCl_3$ and $PbCl_2$ aqueous solutions. The metal loading on the solid was determined by XRF.

Treatment group 3: PET/HCl system:

A) Another 6 suspensions of 10 mg of cryo-milled PET is suspended in 10 mL of 0.1 M HCl were left to equilibrate overnight. The solid and liquid phase extraction was performed via decantation method. Solid component was analysed by infrared spectroscopy and the liquid component by bench top NMR.

B) Three from the above six suspensions were treated in the microwave at 75 C for 15 minutes. Solid and liquid components were separated. The solid component was analysed by IR and the liquid component by bench top NMR.

C) Approximately 5 mg of the solid extracted from in A and in B was used for adsorption of heavy metals from $FeCl_3$ and $PbCl_2$ aqueous solutions. The metal loading on the solid was determined by XRF.

A total of 18 treatment groups were prepared and were used for following bioassay experiments.

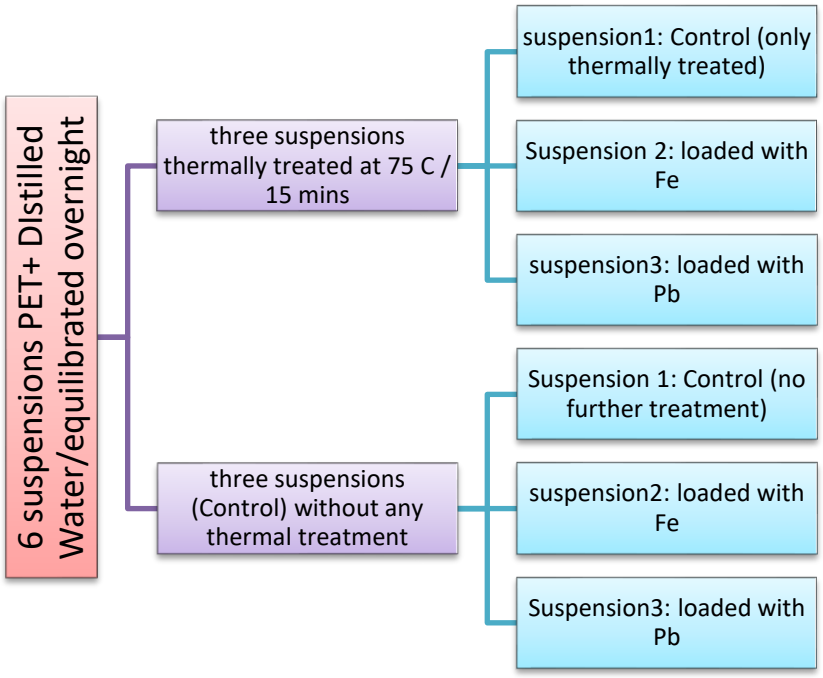

Figure 4.2: Treatment group 1; Cyro-milled PET suspended in distilled water, solid and liquid phase extraction was done for 6 final thermally treated and untreated samples, solids and liquid phases were separately analysed through IR and NMR

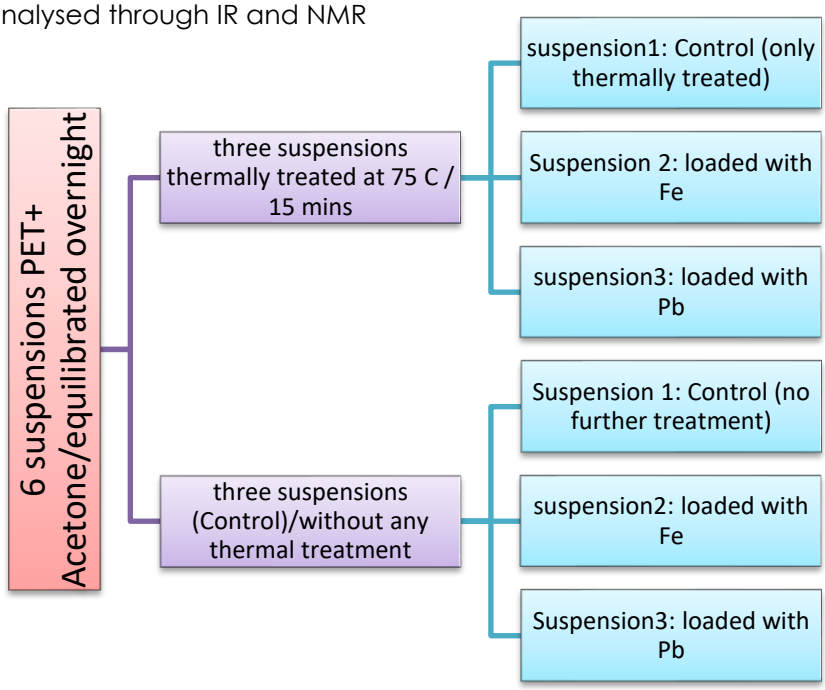

Figure 4.3: Treatment group2 ; Cryo-milled PET suspended in Acetone, solid and liquid phase extraction was done for 6 final thermally treated and un treated samples, solids and liquid phases were separately analysed through IR and NMR

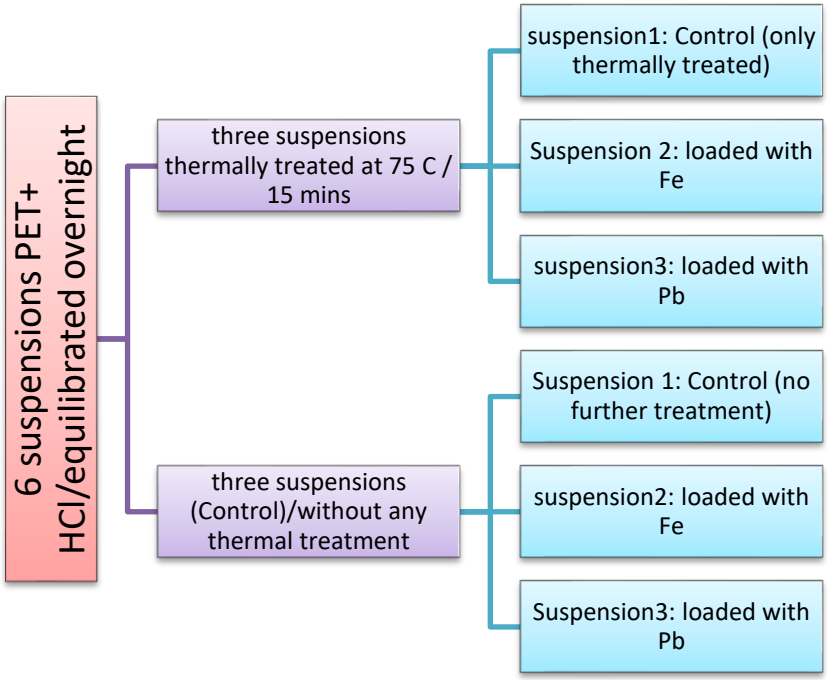

Figure 4.4: Treatment group 3: Cryo-milled PET suspended in HCl , solid and liquid phase extraction was done for 6 final thermally treated and un treated samples, solids and liquid phases were separately analysed through IR and NMR

4.2.2. Bioassay

The fresh culture of *Daphnia* was purchased from Blades Biological Ltd. UK. Cultures were reared in optimized lab conditions to be used for subsequent bioassay experiments. Briefly, *Daphnia* culture was maintained at water temperature of 18 ± 1 °C, photoperiod of 16 h light: 8 h dark. Glass bottled mineral water was used as culture medium. Food was *Chlorella vulgaris* provided every day from Monday to Friday, 3×10^5 cells/mL/daphnia.

Three bioassays were performed simultaneously for each treatment group:

with the thermally treated PET particles extracted and dried from three PET suspensions in water, HCL and Acetone. The bioassays were started with juvenile daphnia (first brood, 48h-72h old).

Ten juvenile Daphnia were exposed to each treatment group in 500 mL glass beakers with 300 mL of test medium. Beakers were covered but allowed air changes. The test medium was refreshed every 24 h, the exposure period was 7 days. Treatments containing PET particles were prepared by serial dilution of a stock solution (5 mg/L of MPs in test medium) into test medium.

Organisms were exposed with a final concentration of 1mg/l of the extracted PET particles as well as heavy metals in the following exposure settings.

Table 4.1: Exposure groups for thermally & non-thermally treated PET particles in three different solvents; Acetone, H2O and HCl

	SOLVENT		
	Acetone	HCL	H2O
OVERNIGHT	PET	PET	PET
	PET + Pb	PET + Pb	PET + Pb
	PET+ Fe	PET+ Fe	PET+ Fe
AFTER	PET	PET	PET
THERMAL	PET+ Pb	PET+ Pb	PET+ Pb
TREATMENT	PET+ Fe	PET+ Fe	PET+ Fe

The following physiological parameters were studied over a period of nine days as effect criteria.

1. The mean of somatic growth per daphnia (Growth indicators taken as body length and body width) was measured under Leica DM750 light microscope at 20X magnification. Images were captured with the attached camera and *imageJ* software was used to record the measurements.

2. Mortality rate (No. of Live organisms/total number of organisms * 100) was recorded by counting the number of live moving daphnia in each treatment group.

3. Beats per minute (BPM) were recorded by counting the number of times hear beats in 10 seconds and then was calculated for one minute by multiplying the recorded number with 6.

4. Reproduction (number of embryos in brood chamber) was recorded by counting the number of embryos in the brood chamber.

Statistical analysis using IBM SPSS was performed to determine the significance of the findings. The analysis was made for the main effects of TIME POINT (Exposure Day), HEAT (PET particles been thermally treated or not), EXPOSURE (PET with metal interaction or without metal interaction) and SOLVENT (HCl, H_2O and Acetone) considered as 'Dependent variables'. Five 'response variables' i) body length, ii) body width ii) mortality iv) Beats per minute (BPM) and v) Reproduction were studied. Since there were more than one independent variable under investigation, a general linear model was used to analyse the relatively normally distributed variables length, width, and BPM (dependent variables). However, for reproduction (dependent) variable, since the data was categorical, general linear model could not be used. Hence, Wald Chi-Square test performed to analyse the reproduction behaviour under different exposure conditions. To understand the interaction effects of statistically significant variable (with p-value < 0.05) estimated marginal

means were calculated during the analysis and then the values were plotted in Microsoft EXCEL. Marginal mean is the mean response for each category of a factor, adjusted for any other variables in the model.

4.3. Results

4.3.1. Chemical analysis

XRF analysis was performed to quantitatively analyze the potential of thermally treated PET particles to adsorb metals on their surface. Concentrations of Pb^{2+} and Fe^{2+} were measured in parts per million (ppm). Results indicate that the adsorption capacity of non-thermally treated PET particles was higher for both metals Pb^{2+} and Fe^{2+} (figure 4.4, 4.5), except for only the treatment group PET + H_2O which showed a higher adsorption rate for Pb^{2+} in thermally treated PET particles (figure 4.4). The results shown in figures 4.4 and 4.5 provide evidence for a significant trend in the behaviour of PET particles when dissolved in different solvents. The experiments investigated the effects of three solvents, HCl, H_2O, and Acetone, on the adsorption behaviour of PET particles. The reduced binding of metal particles dissolved in HCl was observed as compared to H_2O and Acetone.

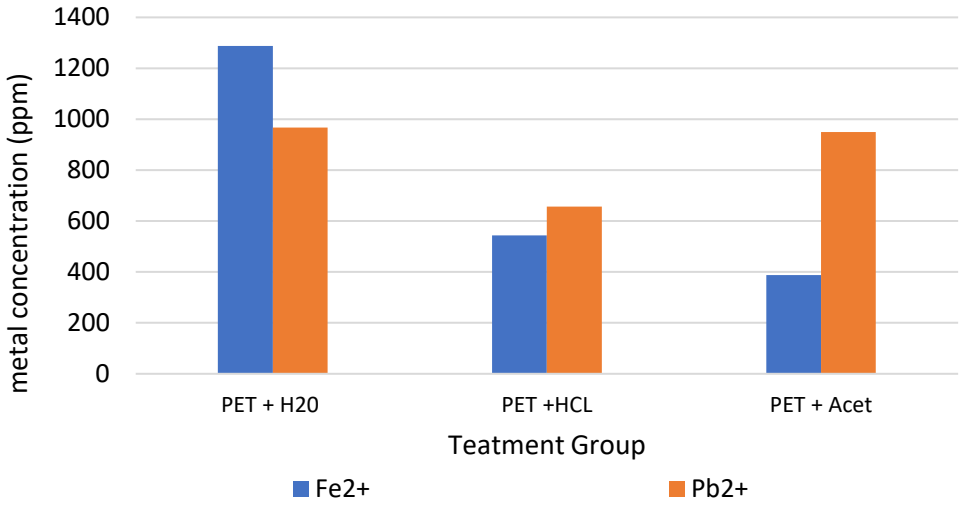

Figure 4.5: Adsorption behaviour of non-thermally treated PET particles for Fe+2 and Pb+2 in H2O, HCl and Acetone solvents.

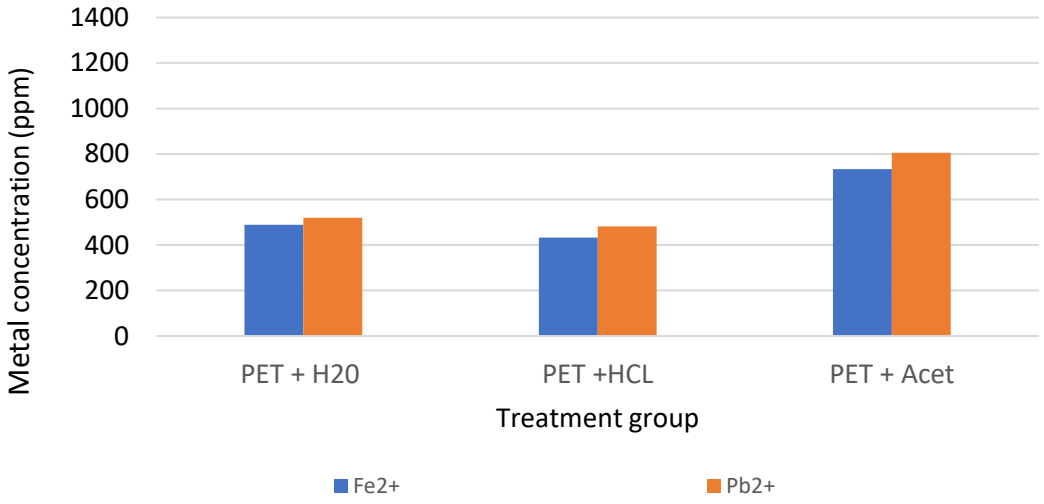

Figure 4.6: Adsorption behaviour of thermally treated PET particles for Fe+2 and Pb+2 in H2O, HCl and Acetone solvents.

Following the PET-metal adsorption analysis, the degradation of thermally treated PET particles in targeted solvents was evaluated by **FT-IR spectroscopy**. Figures 4.6 and 4.7 represent the FT-IR spectra of cryomilled PET particles mixed with H_2O, HCl

and Acetone. The FT-IR spectra for all treatment groups, with or without heat treatment were found to be very similar in terms of peak values suggesting that the given heat treatment as well as the presence of investigated solvents does not completely alter the chemistry of PET particles. However, the peak intensities varied slightly which means there is some loss of relative abundance of specific functional groups in examined PET particles.

Figures 4.6 and 4.7 show the characteristic peaks for PET as described by Chen et al., 2021. According to this, the absorption bands observed at 3100–2800 cm^{-1} are attributed to aromatic and aliphatic –C–H bond stretching. At 2965 cm^{-1}, absorption associated to the symmetrical stretch of the C–H bond was observed, with a higher intensity for the PET in HCL and H_2O with no thermal treatment. A broad band centered at 3412 cm^{-1} probably due to the water absorption was observed in non-thermally treated group PET+HCL and PET+H_2O whereas this band was not observed in any of the thermally treated groups. The peak at 1713 cm^{-1} is associated to the ester carbonyl bond stretching, the band at 1244 cm^{-1} has been attributed to the stretching vibration of the ester group, –C–O–C = O (Chen et al., 2012). A band intensity in this region is seen to be lower for all treatment groups compared to the milled PET control. The absorption band at 1093 cm^{-1} is attributed to the methylene group while the region 1050–990 cm^{-1} is attributed to the in-plane bending of the C–H bonds of a 1, 4-di-substituted aromatic ring (Chen et al., 2012). Among the two treatment groups, it is evident that the loss of band intensities was more noticeable in the thermally treated PET treatment groups as compared to non-thermally treated ones. This suggests that the heat treatment resulted in partial degradation of PET particles.

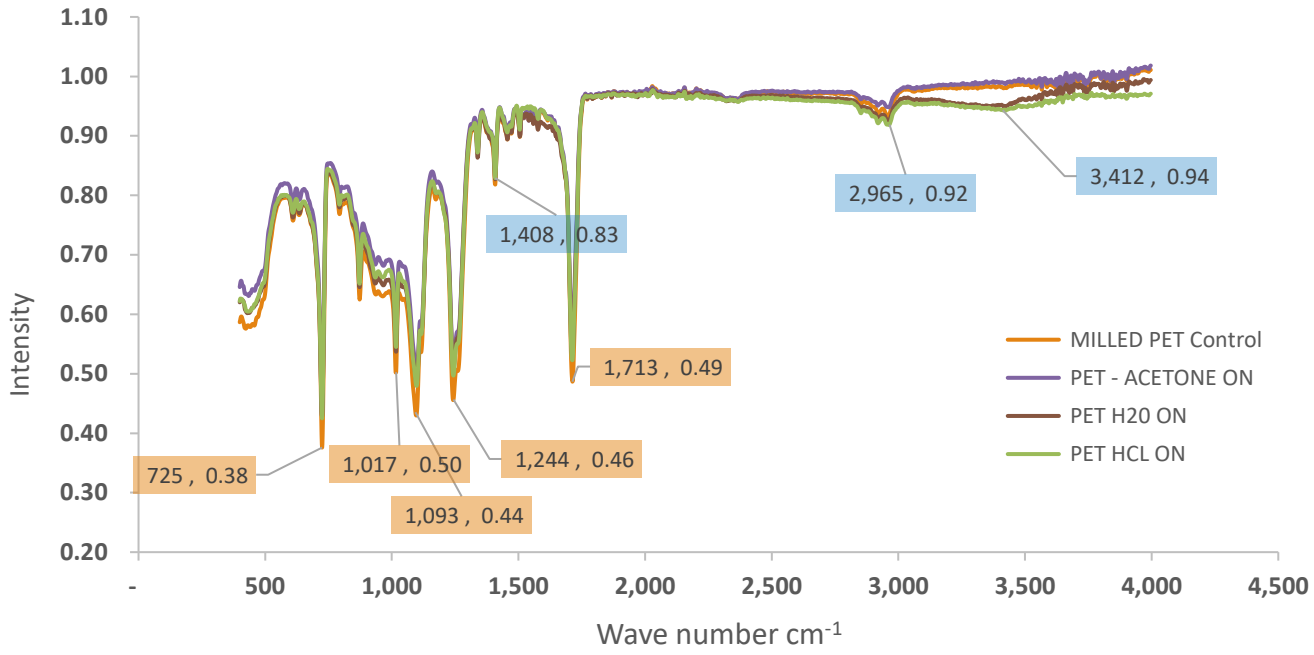

Figure 4.6: Comparison of IR spectra generated for non-thermally treated cryo-milled PET particles in different solvents.

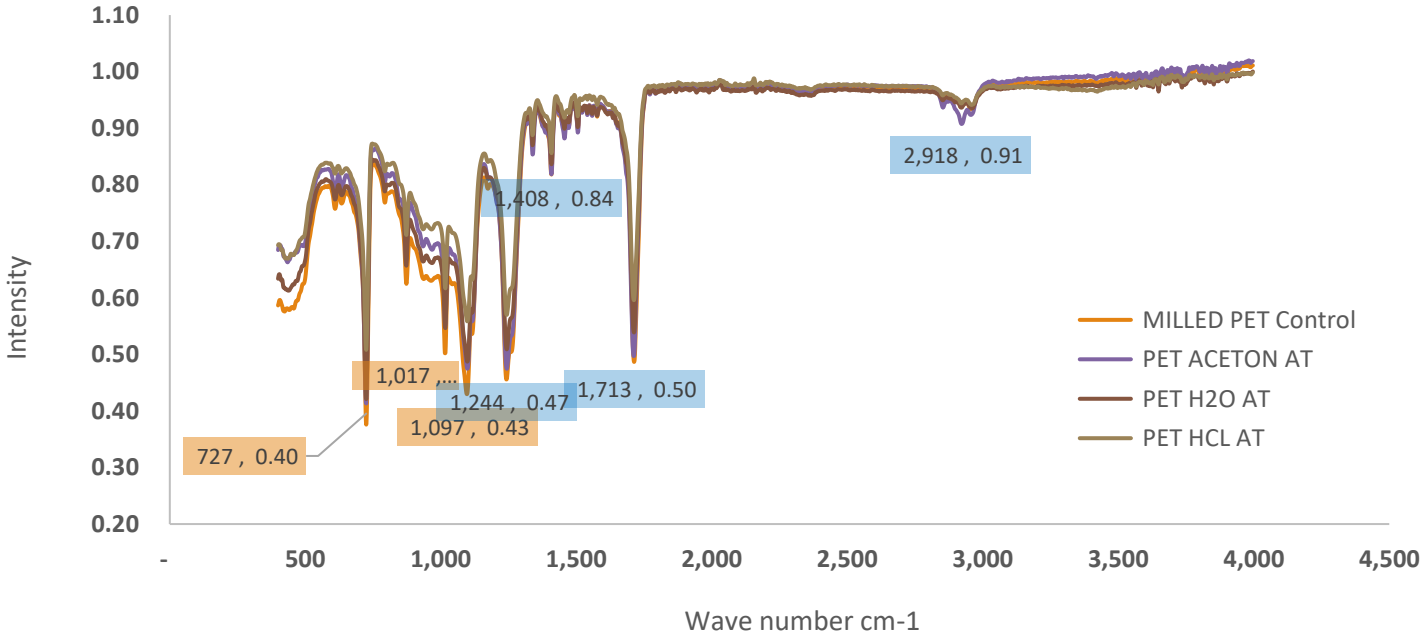

Figure 4.7: Comparison of IR spectra generated for cryo-milled PET particles in different solvents after heat treatment.

^{1}H NMR analysis was employed to further investigate the degradation of PET particles in present experimental conditions. The PET degradation products or functional groups released as a result of thermal treatment in the solvents were anticipated to be observed during the analysis. Table 4.2, 4.3 and 4.4 represent the functional groups observed in the NMR spectra generated for H_2O, HCl and Acetone extracted from the PET suspensions with or without thermal treatment. The results showed that the cryo-milled PET microparticles were not completely degraded, irrespective of the solvent used.

A group of broad signals centered at 1.3 and 1.8 ppm due to the CH and CH_2 protons of the polymer chain, respectively were also observed which also indicates that some degradation has occurred as a result of thermal treatment.

Table 4.2: list of functional groups observed in ^{1}HNMR spectra of Acetone solvent extracted from PET+Acetone suspension with or without heat treatment, compared to the Acetone reference spectra.

	PET+ ACETONE OVERNIGHT		**PET+ ACETONE AFTER THERMAL TREATMENT**		**ACETONE REFERENCE**	
1	Ppm	Functional Groups	Ppm	Functional Groups	Ppm	Functional Groups
2	0.43.1.13,1.91	Alkanes	0.21, 0.41, 0.85, 1.10, 1.32, 1.88	Alkanes	1.84	Alkanes
4	2.71-3.06	Alkynic	2.39, 2.56, 2.69	Carbonyl	2.98	Alkynic
5	3.27,3.38,3.59	Ether	3.01, 3.13, 3.25,3.35,	Ether	4.32	Ester
6	4.36, 4.19	Ester	3.81, 4.38	Ester		

Table 4.3: list of functional groups observed in ^{1}HNMR spectra of HCl solvent extracted from PET+HCl suspension with or without heat treatment, compared to the HCl reference spectra.

	PET+ HCL OVERNIGHT		PET+ HCL AFTER THERMAL TREATMENT		HCL REFERENCE	
1	Ppm	Functional Groups	Ppm	Functional Groups	Ppm	Functional Groups
2	2.68	Carbonyl	2.8	Alkynic		
3	3.4	Ether	3.5	Ether		
4	4.6	Amide	4.6	Methoxy/alkoxy	4.6	R2C=CH2
5	5.8	Vinylic	5.5	Vinylic	5.6	Vinylic
6	6.1	Aromatic (Benzene ring)	5.9	Vinylic	5.8	Vinylic
7	7.0	Aromatic (Benzene ring)	6.02,6.3	(Aromatic (Benzene ring)		

Table 4.4: list of functional groups observed in ^{1}HNMR spectra of H_2O solvent extracted from PET+ H_2O suspension with or without heat treatment, compared to the H_2O reference spectra.

S. NO	PET+ H2O OVERNIGHT		PET+ H2O AFTER THERMAL TREATMENT		H2O REFERENCE	
1	Ppm	Functional Groups	Ppm	Functional Groups	Ppm	Functional Groups
2	3.11	Ether	3.14	Ether	2.85	Ar-CH
3	3.56	Ether	3.43	Ether	3.41	C-H-O
4	4.66	R2C=CH2	4.68	R2C=CH2	4.63	R2C=CH2
5	5.90	RCH=CHR	5.30	R2C=CH2	5.45	RCH=CHR
6	6.35	Aromatic	5.84	RCH=CHR	5.78	RCH=CHR
7	7.00	Aromatic	6.14	Aromatic		

The results obtained from NMR analysis are consistent with those obtained from FT-IR, which also indicated the loss of some functional groups (reduced peak intensity compared to standard) in all experiment groups.

4.3.2. Bioassay Experiment

A bioassay was performed to determine the impact of thermally treated PET particles at exposure concentration of 1mg/l on *Daphnia pulex* under laboratory conditions for over 7 days. The results for the studied parameters are reported below.

4.3.2.1. Effect of thermally treated PET microplastics in different exposure groups on Survival rate of Daphnia *pulex*

The mortality rate was observed to be the most significant consequence of PET exposure across all treatment groups. According to the results obtained, the untreated PET particles exhibited relatively higher toxicity, resulting in higher mortality rates compared to the thermally treated particles. Specifically, in the UT PET(H2O)+ Fe^{2+} group, no daphnia were able to survive beyond day 3 (figure 4.8(c)). Additionally, among the subgroups of untreated PET, higher mortality rates were observed in those loaded with Fe^{2+} and Pb^{2+} (UT PET(Acetone)+Pb^{2+}=90%, followed by UT PET(Acetone)+ Fe^{2+}=60% as compared to untreated PET without metal interaction (except for the group UT PET(Acetone)=90%). Among the thermally treated PET subgroups, the highest mortality rate was observed in the MTT PET(Acetone)+Pb^{2+} group (80%), while the lowest was observed in the MTT PET(HCl)+Fe^{2+} group (10%). This indicates that the partial degradation of PET particles resulting from thermal treatment is responsible for reduced toxicity and consequently

a lower mortality rate. Acetone appears to have an effect on the degradation and higher toxicity of PET particles, as observed among the three solvents studied.

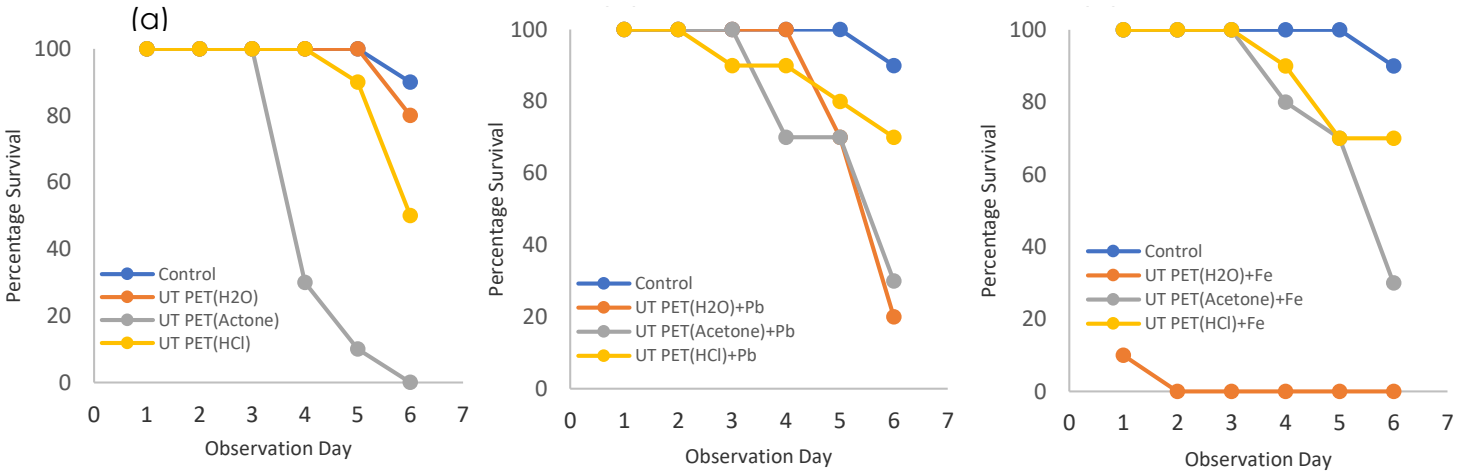

Figure 4.8: The survival percentage plotted for exposure groups of untreated PET (UT PET) particles a) with no metal interaction, B) with $Pb^{2}$and c) with Fe^{2+} $^{+}$; x-axis represents the observation day, y-axis represents the percentage survival.

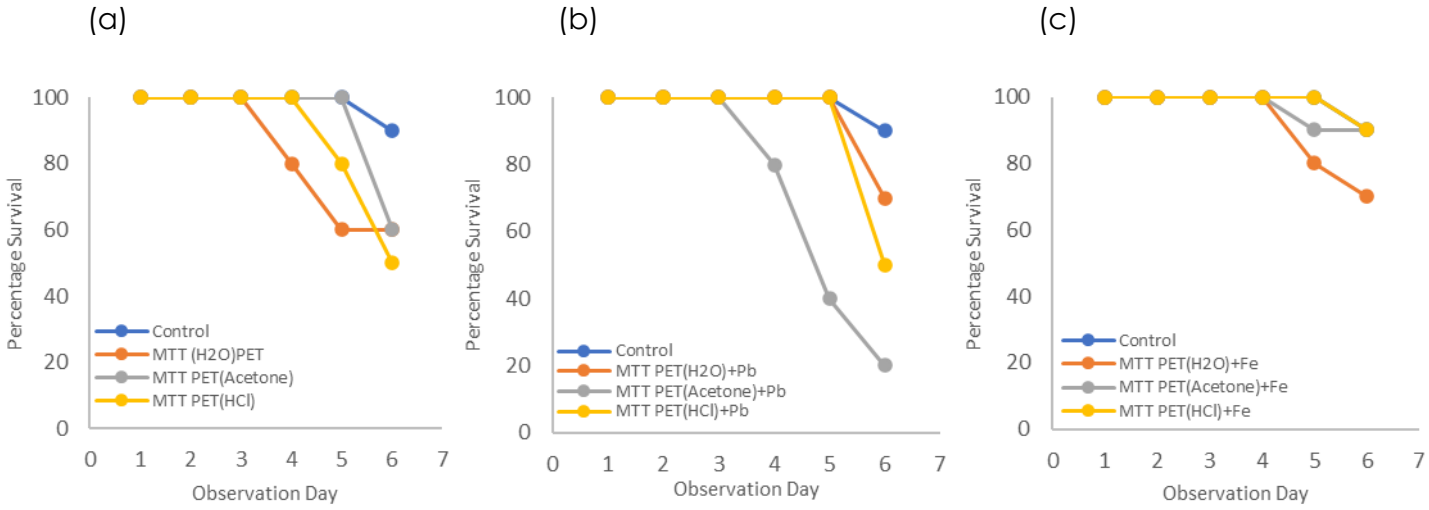

Figure 4.9: The survival rate plotted for exposure groups of microwave-assisted thermally treated PET (MTT PET) particles a) with no metal interaction, B) with Fe^{2+} and c) with Pb^{2+}; x-axis represents the observation day, y-axis represents the percentage survival.

4.3.2.2. Effect of thermally treated PET microplastics in different exposure groups on growth of Daphnia *pulex* (body length and body width)

A) Body Length:

From the four main effect variables i) Thermal treatment of PET particles ii) Time Point iii) Solvent, iv) Metal interaction (loading of Fe and Pb with PET), the thermal treatment had an indirect effect on body length ($p=0.005$), whereas the rest of variables did not show any significant effect on daphnia body length. The interpretation is made from the R squared value obtained for the model (general linear model) which is less than 1 (R square value between 1 and 5 shows the respective variables have significant effect on response variables (as shown in Figure 4.8).

Among all the treatment groups, higher body length was observed for thermally treated exposure groups. The highest value recorded for thermally treated PET+PB in H_2O solvent was 1.728 mm with the increase of 0.27 mm at day 7, followed by thermally treated PET without any metal exposure with the increase of 0.33 mm (table 4.5). Approximately similar trend was observed for the other exposure groups of Acetone and HCl solvents (table 4.6, 4.7).

Table 4.5: Average body length of daphnia *pulex* after being exposed to 7 exposure groups of cryomilled PET+H2O, measured in mm, for 7 days.

TREATMENT GROUP (PET+H_2O)	EXPOSURE DAY BODY LENGTH (MM)					
	Day2	**Day3**	**Day4**	**Day5**	**Day6**	**Day7**
CONTROL	1.561	1.438	1.497	1.491	1.525	1.6
UT PET(H_2O)+ PB^{2+}	1.397	1.594	1.312	1.447	1.7	1.35
MTT PET(H_2O)+ PB^{2+}	1.591	1.589	1.49	1.714	1.772	1.728
UT PET(H_2O)+ FE^{2+}	1.55	-	-	-	-	-
MTT PET(H_2O)+ FE^{2+}	1.294	1.442	1.387	1.344	1.418	1.47
UT PET(H_2O)	1.455	1.493	1.418	1.555	1.594	1.507
MTT PET(H_2O)	1.383	1.562	1.75	1.571	1.52	1.65

Table 4.6: Average body length of daphnia *pulex* after being exposed to 7 exposure groups of cryomilled PET(Acetone)ACETONE, measured in mm, for 7 days.

TREATMENT GROUP PET+ACETONE	EXPOSURE DAY BODY LENGTH (MM)					
	Day2	**Day3**	**Day4**	**Day5**	**Day6**	**Day7**
CONTROL	1.561	1.438	1.497	1.491	1.525	1.371
MTT PET(Acetone)	1.399	1.443	1.493	1.395	1.352	1.58
UT PET(Acetone)	1.375	1.55	1.487	1.629	1.367	1.496
MTT PET(ACETONE)+FE^{2+}	1.398	1.545	1.518	1.448	1.422	1.477
UT PET(ACETONE)+FE^{2+}	1.35	1.441	1.485	1.458	1.416	1.47
MTT PET(ACETONE)+PB^{2+}	1.447	1.337	1.459	1.484	1.555	1.557
UT PET(ACETONE)+PB^{2+}	1.404	1.346	1.362	1.387	1.471	1.418

Table 4.7: Average body length of daphnia *pulex* after being exposed to 7 exposure groups of cryomilled PET+HCl, measured in mm, for 7 days.

TREATMENT GROUP PET+HCL	**EXPOSURE DAY BODY LENGTH (MM)**					
	Day2	**Day3**	**Day4**	**Day5**	**Day6**	**Day7**
CONTROL	1.561	1.438	1.497	1.491	1.525	1.371
MTT PET(HCL)	1.47	1.46	1.41	1.42	1.27	1.52
UT PET (HCL)	1.44	1.50	1.41	1.46	1.50	1.53
MTT PET(HCL)+FE^{2+}	1.52	1.47	1.41	1.42	1.43	1.65
UT PET(HCL)+FE	1.54	1.47	1.55	1.55	1.55	1.63
MTT PET(HCL)+PB^{2+}	1.32	1.57	1.54	1.53	1.46	1.68
UT PET(HCL)+PB^{2+}	1.42	1.43	1.49	1.42	1.43	1.36

To reduce the complexity of the responses shown by daphnia against multiple treatment groups (taken as independent variables), the statistical analysis estimated the significance of each independent variable (i)Thermal treatment of PET particles, ii) Solvent used during the thermal treatment process and iii) Interaction of Fe and Pb ions with thermally and non-thermally treated PET particles) individually against each response variable(i)*Daphnia* Body length, ii)*Daphnia* Body width iii)Heart rate iv)Reproduction and v)Survival rate). In the given analysis, the estimation of significance (p value) for the Solvent variable is based on the overall impact of all subgroups of respective solvents. Similarly, p value for the impact of variable thermal treatment means the overall impact of all subgroups with thermally treated PET or without thermally treated PET.

Likewise, significance of metal interaction means the impact of Pb^{2+} loading on

thermally or non-thermally PET in three studied solvents. Table 4.8 represents the p values for each of the studied variables. According to this table, the only variable 'Thermal treatment' had p-value equal to 0.05 which suggests that there is marginally significant effect of thermal treatment on PET particles that results in difference in daphnia body length over 7 days. However, the vale R squared = 0.18 (which is less than 1) indicates that overall, all the studied variables have almost no effect on Daphnia's growth in terms of mean body length in given period of time.

Table 4.8: ANOVA table for the main effect of LENGTH (Dependent variable), the green color

	Type III Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	.736[a]	9	.082	1.938	.044s
Intercept	1653.900	1	1653.900	39169.038	<.001
TIME POINT	.228	5	.046	1.079	.370
SOLVENT	.131	2	.065	1.548	.213
THERMAL TREATMENT	.337	1	.337	7.983	.005
METAL INTERACTION	.027	1	.027	.650	.420
Error	39.733	941	.042		
Total	2084.827	951			
Corrected Total	40.470	950			

a. R Squared = .018 (Adjusted R Squared = .009)

highlighted row indicates the significant variable with p=0.005, whereas all other variables are non-significant with p>0.005.

To analyse the directional relationship between the thermal treatment and the body length, estimated marginal means were calculated for the marginally significant effect (p=0.005) of thermal treatment, as indicated in table 4.10. Consequently, the interaction effect of this variable was assessed by plotting the marginal means. The results of the plot revealed that organisms exposed to thermally treated PET particles had a relatively longer body length compared to those not exposed to thermal treatment (figure 4.10).

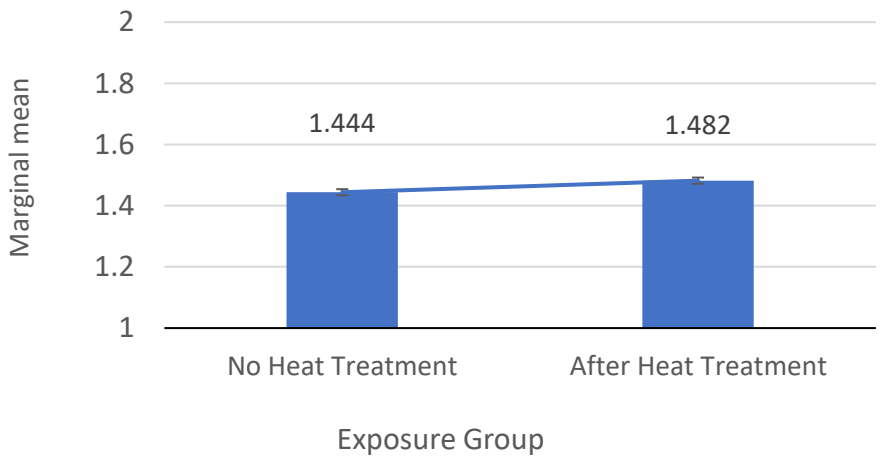

Figure 4.10: Estimated Marginal means of daphnia body length plotted for the main effect of HEAT (Thermally treated vs non-thermally treated PET particles), x-axis represents the exposure group and y-axis represents marginal means; a general trendline is shown to show the interaction across the exposure groups.

B) Body Width

Among the three solvents, the highest body width was observed in the organisms exposed to PET particles extracted from HCl suspension, followed

by Acetone and then water.

From the two PET and metal combinations, MTT PET(HCl)+Pb^{2+} showed a higher body width of 0.95 mm at day 7. Least body width was observed in treatment group UT PET(H_2O).

Table 4.9: Average body width of daphnia *pulex* after being exposed to 7 exposure groups of cryomilled PET+ H_2O measured in mm, for 7 days.

TREATMENT GROUP (PET+H_2O)	**EXPOSURE DAY BODY WIDTH (MM)**					
	Day2	**Day3**	**Day4**	**Day5**	**Day6**	**Day7**
CONTROL	0.792	0.737	0.743	0.73	0.689	0.86
UT PET(H_2O)+ PB^{2+}	0.83	0.95	0.869	0.81	0.768	0.731
MTT PET(H_2O)+ PB^{2+}	0.95	0.703	0.86	0.824	0.747	0.818
UT PET(H_2O)+ FE^{2+}	0.84	-	-	-	-	-
MTT PET(H_2O)+ FE^{2+}	0.741	0.654	0.796	0.779	0.761	0.781
UT PET(H_2O)	0.837	0.569	0.821	0.648	0.656	0.621
MTT PET(H_2O)	0.656	0.646	0.745	0.692	0.686	0.7

There was a minimal yet noticeable impact of TIME POINT (p=0.011), SOLVENT (p=0.001) and METAL INTERACTION (p=0.048) on the body width measurements. Nevertheless, the overall interpretation from the model suggests that the combined impact of all the exposure conditions is rather insignificant based on R-squared value which is less than 0.1 (see table 5.11)

Table 4.10: Average body width of daphnia *pulex* after being exposed to 7 exposure groups of cryomilled PET+ Acetone measured in mm, for 7 days.

TREATMENT GROUP (PET+ACETONE)	**EXPOSURE DAY BODY WIDTH (MM)**					
	Day2	**Day3**	**Day4**	**Day5**	**Day6**	**Day7**
CONTROL	0.792	0.737	0.743	0.73	0.689	0.86
MTT PET(ACETONE)	0.787	0.729	0.616	0.797	0.7	0.822
UT PET(ACETONE)	0.704	0.757	0.7755	0.717	0.81	0.851
MTT PET(ACETONE)+ FE^{2+}	0.823	0.786	0.795	0.703	0.796	0.865
UT PET(ACETONE)+ FE^{2+}	0.792	0.773	0.787	0.72	0.882	0.803
MTT PET(HCL)+ PB^{2+}	0.829	0.742	0.772	0.758	0.815	0.907
UT PET(H_2O)+ PB^{2+}	0.78	0.814	0.812	0.794	0.633	0.872

Table 4.11: Average body width of daphnia *pulex* after being exposed to 7 exposure groups of cryomilled PET+ HCl measured in mm, for 7 days.

TREATMENT GROUP (PET+HCL)	**EXPOSURE DAY BODY WIDTH (MM)**					
	Day2	**Day3**	**Day4**	**Day5**	**Day6**	**Day7**
CONTROL	0.792	0.737	0.743	0.73	0.689	0.86
UT PET(HCl)+ PB^{2+}	0.714	0.84	0.792	0.813	0.884	0.892
MTT PET(HCL)+ FE^{2+}	0.835	0.819	0.818	0.795	0.784	0.846
UT PET(HCl)+ FE^{2+}	0.856	0.819	0.889	0.866	0.861	0.824
MTT PET(HCl)+ PB^{2+}	0.85	0.869	0.847	0.874	0.803	0.95
UT PET(HCl)	0.753	0.772	0.808	0.779	0.776	0.765

Table 4.12: ANOVA table for the main effect of WIDTH (Dependent variable), the green colour highlighted rows indicate the significant variable with p<0.05, whereas all other variables are non-significant with p>0.05.

	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1.496[a]	9	.166	8.097	<.001
Intercept	471.556	1	471.556	22967.605	<.001
TIME POINT	.306	5	.061	2.978	.011
SOLVENT	1.007	2	.503	24.520	<.001
THERMAL TREATMENT	.024	1	.024	1.189	.276
METAL INTERACTION	.081	1	.081	3.928	.048
Error	19.320	941	.021		
Total	598.578	951			
Corrected Total	20.816	950			

a. R Squared = .072 (Adjusted R Squared = .063)

The below gives the marginal means for the levels of these factors.

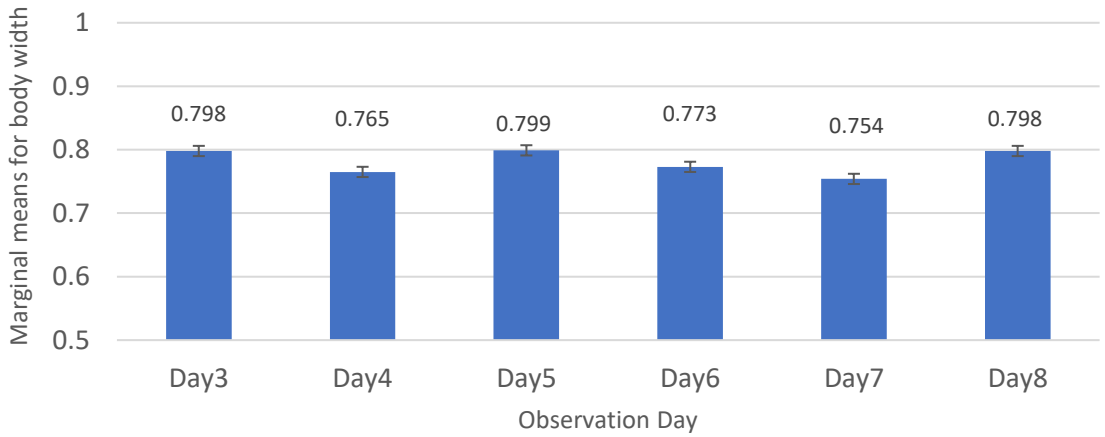

Figure 4.11: Estimated Marginal means of daphnia body width plotted for the main effect of TIME POINT (observation day), x-axis represents the observation day and y-axis represents marginal means.

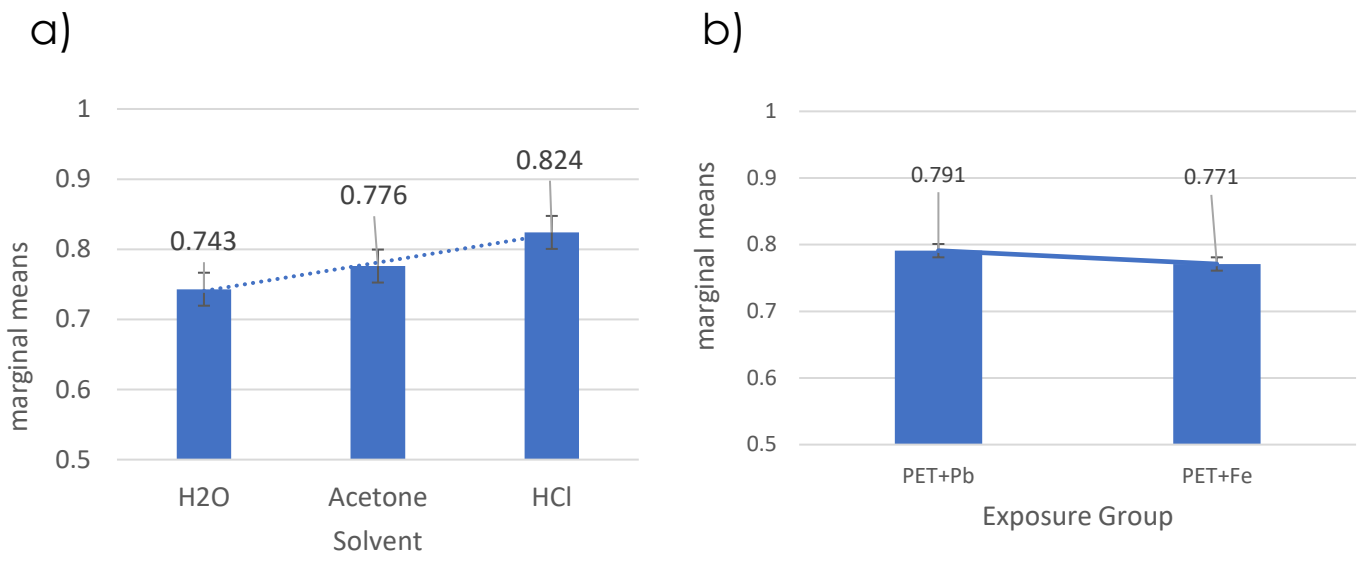

Figure 4.12 (a,b) Estimated Marginal means of daphnia body width plotted for the main effect of a) SOLVENT (H2O, ACETONE and HCl), x-axis represents the Solvent and y-axis represents marginal means, b) EXPOSURE GROUP (with or without metal interaction)), x-axis represents the exposure group and y-axis represents marginal means

No general trend was observed for marginal means of body width over 7 days as shown in figure 4.13. However, the marginal means did show a general trend for solvent variable (4.14 a) with highest body width observed in the daphnia exposed to PET particles which were suspended in HCl solvent during the thermal treatment process. This is followed by the Acetone based PET particles and lastly the H_2O suspended ones (figure 4.14 a). Similarly, the general trend line (in figure 4.14 b) indicates higher body width measurements for PET with Pb^{2+} exposure group as compared to PET with Fe^{2+}.

4.3.2.3. Effect of thermally treated PET microplastic particles in different exposure groups on Heart rate of Daphnia *pulex*.

The impact of thermally treated PET particles in three studied solvents as well as with the combination of Pb^{2+} and Fe^{2+} on the heart rate can be clearly observed as the R squared value approaches 1, indicating that a large percentage of the variability in the heart rate is accounted for by these independent variables. In other words, the independent variables have a substantial influence on the heart rate and explain a significant portion of its variation. The overall impact of all exposure groups of PET extracted from HCl suspension exhibited the highest heart rate, as compared to the exposure groups of PET extracted from H_2O suspension which showed the lowest heart rate. Throughout the observed period, there was a consistent trend of increasing heart rate in all exposure groups (PET(H_2O), PET(HCl)), except for the PET(ACETONE) exposure group, which experienced a significant decrease in heart rate on day 7.

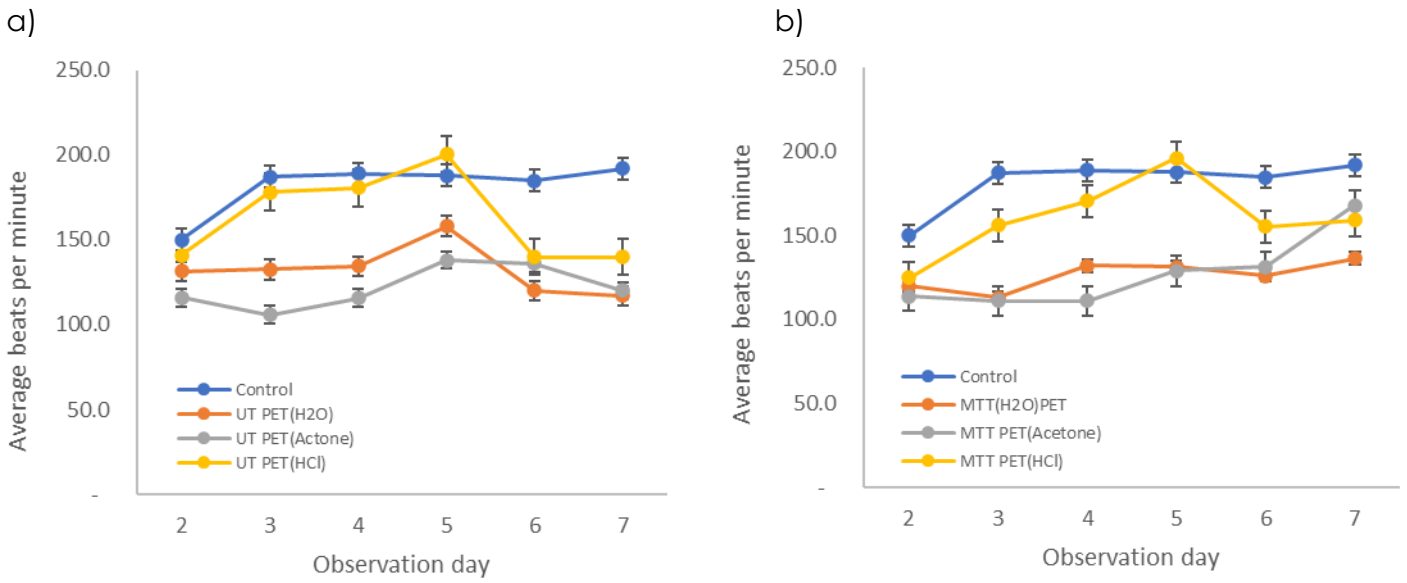

Figure 4.13: The heart rate plotted for exposure groups of **a)** Un treated PET (UT PET) particles with no metal interaction vs **b)** microwave-assisted thermally treated PET (MTT PET) particles with no metal interaction, x-axis represents observation day; y-axis represents the heart rate in terms of average of beats per minute for each group.

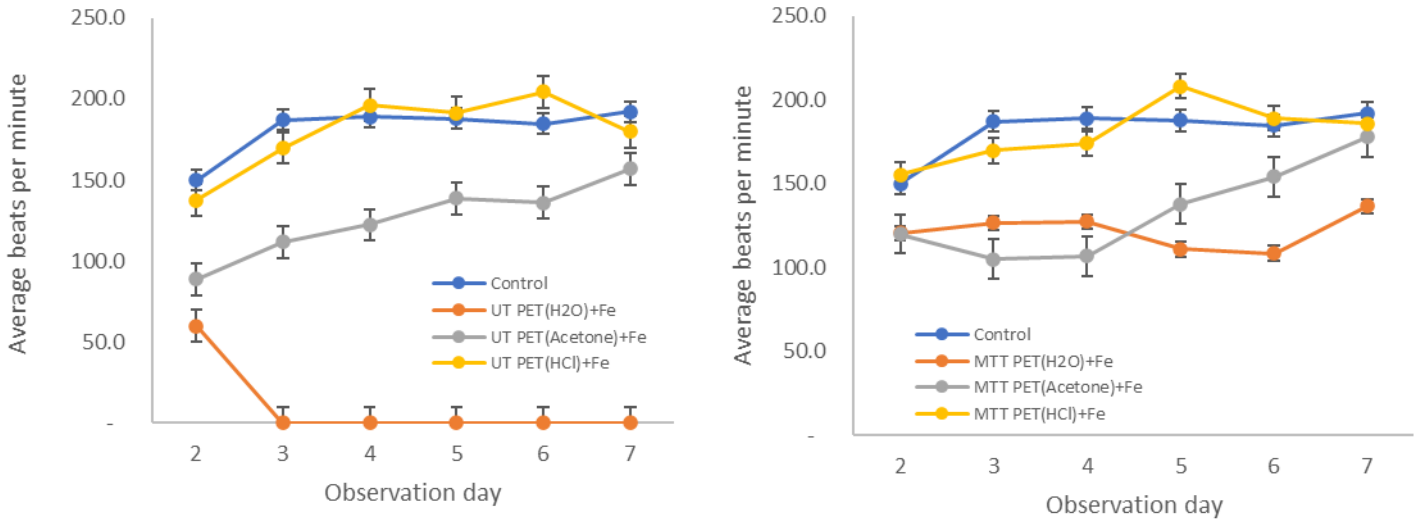

Figure 4.14: The heart rate plotted for exposure groups of **a)** Un treated PET (UT PET) particles with Fe^{2+} metal interaction, **b)** microwave assisted thermally treated PET (MTT PET) particles with Fe^{2+} metal interaction, x-axis represents the observation day, y-axis represents the heart rate in terms of average of beats per minute for each group.

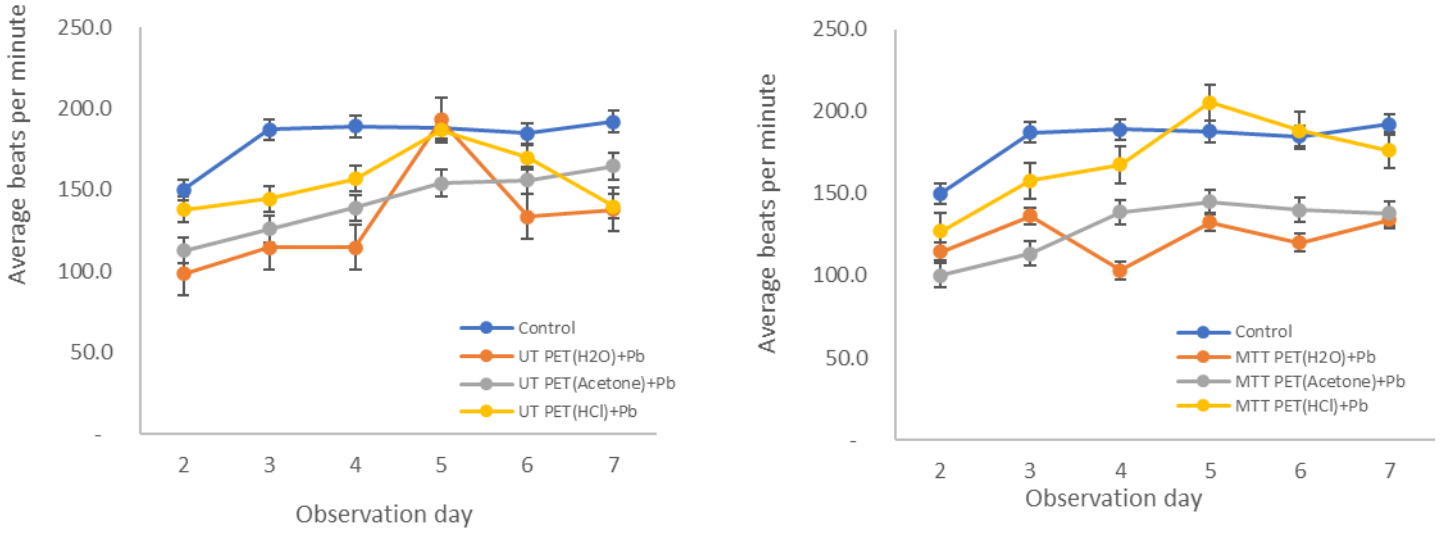

Figure 4.15: The heart rate plotted for exposure groups of **a)** Un treated PET (UT PET) particles with Pb^{2+} metal interaction, **b)** microwave assisted thermally treated PET (MTT PET) particles with Pb^{2+} metal interaction, x-axis represents the observation day, y-axis represents the heart rate in terms of average of beats per minute for each group.

The findings from the results (Figure 4.15, 4.16, 4.17) clearly indicate that the heart rate was consistently lower in all treatment groups compared to the control group. Although there was not a significant disparity in the heartbeat rate between the thermally treated and untreated exposure groups, an interesting trend was observed in the HCl based PET particles exposure group. In this group, the heartbeat rate was closest to that of the control group compared to all other treatment groups. The lowest heart rate was recorded in daphnia that were exposed to PET particles suspended in Acetone during the treatment process. A general linear model was used to analyse BPM for main effects of thermal treatment, Solvent, Metal interaction and Time point.

The analysis of variance (ANOVA) (Table 4.16) represents the significance of the main effects.

Table 4.13: ANOVA table for the main effect of BPM (Dependent variable), the green colour highlighted rows indicate the significant variable with p<0.05, whereas all other variables are non-significant with p>0.05.

	Type III Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	935504.527[a]	9	103944.947	91.125	<.001
Intercept	14736017.381	1	14736017.381	12918.632	<.001
TIME POINT	184345.478	5	36869.096	32.322	<.001
SOLVENT	715360.592	2	357680.296	313.568	<.001
THERMAL TREATMENT	3739.056	1	3739.056	3.278	.071
METAL INTERACTION	18624.909	1	18624.909	16.328	<.001
Error	1055128.421	925	1140.679		
Total	19695625.000	935			
Corrected Total	1990632.948	934			

a. R Squared = .470 (Adjusted R Squared = .465)

A significant effect of TIME POINT, SOLVENT and METAL INTERACTION was observed with p<0.001 for all these factors (table 4.16). Moreover, a higher R squared value obtained from the model (0.470) as shown in table 4.16 indicates a stronger relationship between the independent variables TIME POINT, SOLVENT and METAL INTERACTION and heart rate. The below gives the marginal means for the levels of the significant fact.

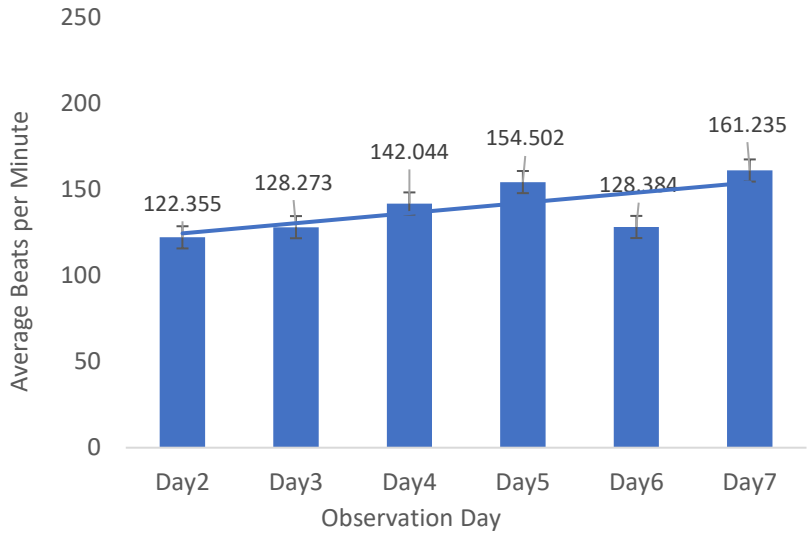

Figure 4.16: Estimated marginal means plotted for the main effect of BPM (dependent variable) against TIME POINT (observation day), x-axis represents the observation day and y-axis represents Average beats per minute.

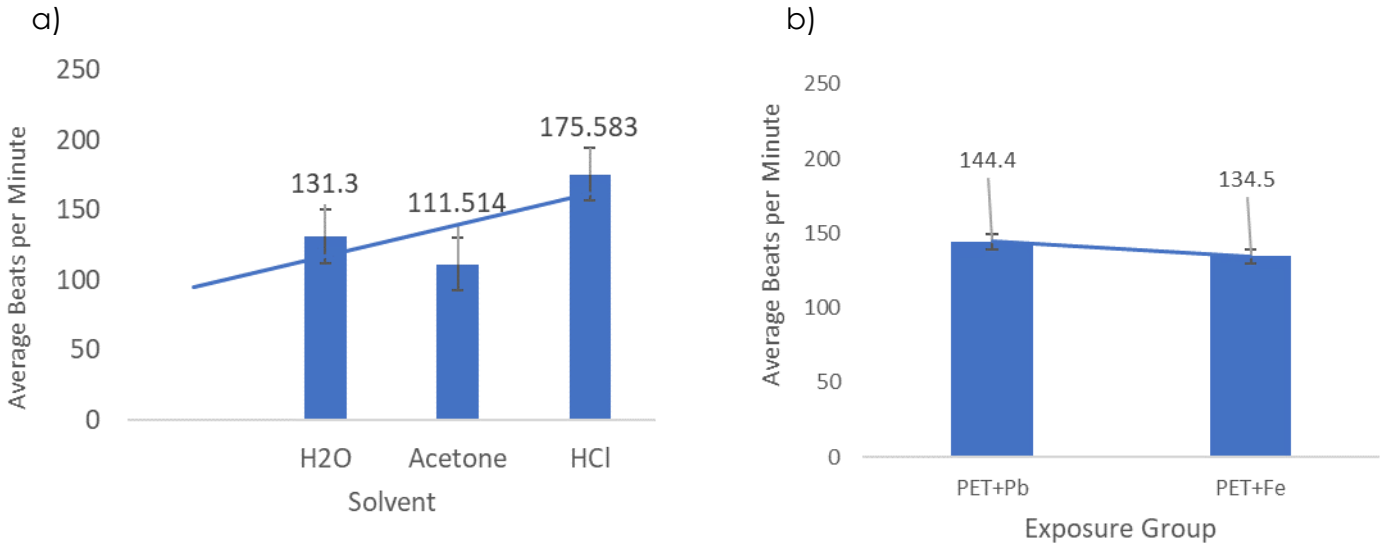

Figure 4.17 (a, b): Estimated marginal means plotted for the main effect of BPM (dependent variable) against a) solvent and b) exposure group; the general trendline is shown which indicates an increase in heart rate over 7 days in (a) whereas slight decrease in (b).

The general trendline in figure 4.18 indicates an overall trend of increasing heartrate with time till the end of observation period. Among the three solvents used during the PET degradation process, HCl based PET particles exposure groups have recorded highest heartrate whereas lowest was observed in Acetone based PET exposure groups (4.19 a). Finally, among the metals studied, Pb^{2+} interacted PET particles exhibited higher heartrate as compared to Fe^{2+} (4.19 b).

4.3.2.4. Effect of thermally treated PET microplastics in different exposure groups on reproductive activity of *Daphnia pulex*.

During the period under investigation, there was an observed increase in reproductive activity among daphnia. When comparing the solvents used, it was found that PET extracted from HCl suspension resulted in a higher production of eggs by the daphnia, while PET from Acetone suspension showed the lowest rate of reproduction. The initial batch of eggs was released on the third day of observation, with all offspring alive. The size of the egg batches varied among the daphnia in each exposure group, with the highest number of eggs being found in the PET+HCl exposure groups and the lowest number of eggs in the PET+Acetone exposure groups. The second batch of eggs began to appear on the sixth day of the experiment. However, due to a mortality rate exceeding 70% in some of the exposure groups by the seventh day, the experiment could not continue long enough to determine if the embryos survived until birth.

For analysis of rate of reproduction, it was attempted to fit a generalised linear model with binary errors for main effects of Heat, Solvent, Exposure and Time point. Table 4.14, below shows the results from analysis of variance, showing the significance of the main effects:

Table 4.14: ANOVA table for the main effect of Reproduction (Dependent variable), the green colour highlighted rows indicate the significant variable with p<0.05, whereas all other variables are non-significant with p>0.05.

	Type III Wald Chi-Square	Df	Sig.
(Intercept)	106.292	1	<.001
SOLVENT	8.337	2	.015
TIME POINT	155.912	5	<.001
METAL INTERACTION	6.916	1	.009
THERMAL TREATMENT	.089	1	.765

Dependent Variable: REPRODUCTION
Model: (Intercept), SOLVENT, TIME, EXPOSURE, HEAT

A significant effect of TIME POINT, SOLVENT, and EXPOSURE was observed on number of broods in each daphnia. The below gives the marginal means for the levels of these factors.

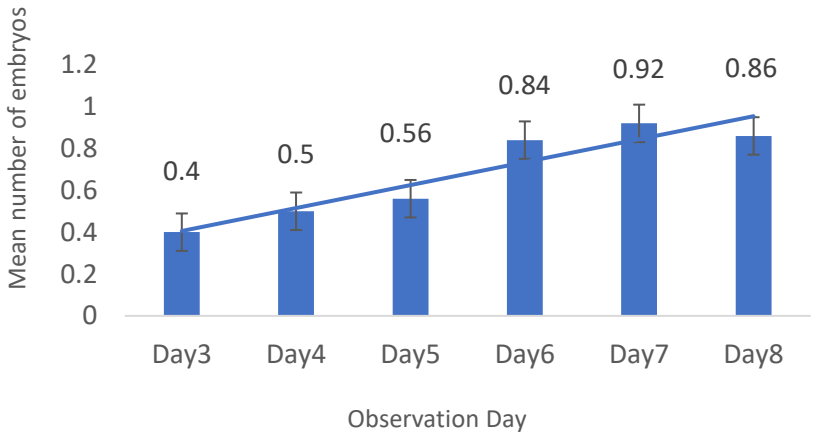

Figure 4.18: Estimated marginal means plotted for the main effect of Reproduction (dependent variable) against TIME POINT (observation day), x-axis represents the observation day and y-axis represents Average number of eggs in brood chamber.

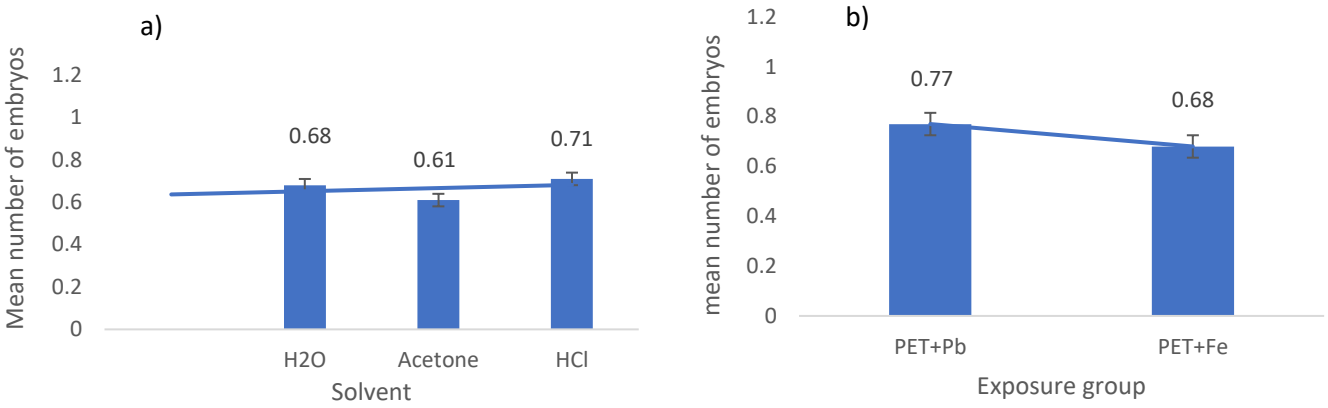

Figure 4.19 (a, b): Estimated marginal means plotted for the main effect of Reproduction (dependent variable) against a) solvent and b) exposure group (b).

Figure 4.18 indicates that the reproductive activity increased for first five days and then decreased. The effect of observation day (Time point) was estimated keeping the other variables (SOLVENT and THERMAL TREATMENT constant. Similarly, when the individual impact of SOLVENT was analysed, it was observed that the Acetone based PET exposure groups had least reproductive activity among the three studied solvents suggesting some effect of Acetone on thermal degradation (4.19a). Among the combine (microplastics+metals) exposure groups, the lower reproductive activity was observed in PET+Fe exposure group (4.19 b).

4.4. Discussion

According to the findings of the experiments conducted for this section of the study, the cryogenically milled PET microplastics did not completely degrade under the intended experimental settings. A small amount of

degradation was observed in the microplastic particles, however none of the three solvents (H_2O, Acetone and HCl) that were chosen had a major effect on degradation. In contrast to the non-thermally treated PET microplastic particles, there were no significant changes observed in the FTIR spectra of PET microplastic particles after undergoing thermal treatment. The peak intensity of the C═O (1703 cm^{-1}) group remained the same before and after the treatment, while the peak of C–O (1093 cm^{-1}) showed a slightly higher intensity after thermal treatment. The broad peak observed between 3600 cm^{-1} and 3300 cm^{-1} in PET particles that were kept overnight without thermal treatment indicates the presence of hydroxyl groups with hydrogen bonds (Silva et al., 2019). It has also been reported that the broad peak between 3000 cm^{-1} and 2500 cm^{-1}, along with the strong peak at 1410 cm^{-1} (Silva et al., 2019)., confirms the existence of carboxylic acid groups in both the treated and untreated experimental groups. Furthermore, upon comparing the bond positions at different time intervals, it was observed that all vibrations related to C–H shifted to lower positions, except for the peaks of out-plane CH_2 vibrations. The peaks of C═O and C–O–C also shifted to lower frequencies. This phenomenon may be attributed to the loss of relevant functional groups along the carbon chains. These results were in consistent with those obtained from the NMR analysis which showed release of similar functional groups. The findings indicate that although the given condition did not completely degrade the PET particles, an increase in treatment

temperature or duration may lead to a greater degree of degradation effects.

The experiment also investigated the effect of microwave-assisted thermal treatment on the potential of PET microplastics to serve as a vector for co-pollutants (heavy metals). It was concluded through the XRF analysis that except for one experiment group (thermally treated PET+H_2O), adsorption of both metals was higher in non-thermally treated PET microplastics. This suggests that the small degradation effects achieved because of thermal treatment have reduced the potential of PET microplastic particles to adsorb Pb^{2+} or Fe^{2+} on its surface. To further evaluate the altered toxicological behaviour of thermally treated PET microplastics on its own as well as in combination with Pb^{2+} and Fe^{2+}, the different treatment groups were exposed to *Daphnia pulex* for period of 7 days. *Daphnia pulex*, a non-selective primary consumer, has an important position in the aquatic food chain and is a well-established model organism in ecotoxicology experiments (An et al., 2021; Jürgens, 1994; Vaz et al., 2021). According to previous studies, *Daphnia* could ingest microplastic particles at a size below 75 µm (Canniff and Hoang, 2018; Schwarzer et al., 2022). Therefore, it was speculated that the micro-PET particles at a size of 20–30 µm could also be ingested by studied *Daphnia* species. Subsequently, the toxic effects of thermally degraded PET particles (in comparison with non-thermally treated PET particles) were investigated on *Daphnia* pulex based on the differences

in physiological parameters. The results showed that the mortality of *Daphnia* pulex was less in response to thermally degraded PET particles with maximum of 60 % in only one of thermally treated PET treatment group. Also, the D*aphnia* were observed to have very slight increased body length in response to the thermally treated PET particles. Bosker et al.,2019 obtained a similar result, in that no significant impact was observed on the number and body length of newborn D. magna offspring following treatment with high MPs. The results report higher mortality in response to microplastic exposure in different treatment groups. This result is consistent with that of Bosker *et al*.,2019 who observed a significant decline in *Daphnia pulex* population biomass due to microplastics exposure. This implies that the lower toxicity effect of thermally treated PET particles might be attributed to the physical or chemical damage induced by micro-wave assisted thermal degradation of cryo-milled PET particles. The PET particles dopped with Fe^{+2} had significantly higher toxicological effects, with 100% mortality at third day of observation in PET+H_2O treatment group. Apart from being ingested, the positively charged PET+ Fe^{+2} might have adsorbed on the surface of daphnids which limited the normal physiological activities of daphnids such as swimming and filtering, which could result in the mortality indirectly. These findings are in consistent with Zheng *et al*.,2021 who concluded the acute toxicity of Fe^{+2} dopped microplastics on *Daphnia*. Other studies also found that surface charge plays an important role in affecting bioavailability and toxicity of microplastic

particles to living organisms, e.g., *Artemia franciscana larvae* (Bergami et al., 2016), *Paracentrotus lividus* (Della Torre et al., 2014), and *Brachionus plicatilis* (Manfra et al., 2017).

The findings for this experiment could further be validated with differential expression analysis and qPCR to investigate the organism's oxidative stress conditions under thermally treated PET exposure. This analysis can give a clearer understanding of reduced toxicological behavior of thermally treated PET. The findings can lead to establishing guidelines and suggestions for targeted thermal treatment of sludge concentrated with microplastics, thus making these microplastics ineffective or non-toxic.

4.5. Conclusion

The data presented in this study suggests that the thermal treatment applied on cryogenically milled PET particles suspended in three different solvents could not have consequential impact on its degradation. However, the partial degradation achieved in this experiment does have some implications in the subsequent bioassays as compared to non-thermally treated PET particles. It was observed that the adsorption capacity of PET microplastic particles for Fe^{2+} and $Pb^{2+}$was reduced after thermal treatment. Moreover, the toxicity of single and combined PET exposure groups was lower for thermally treated PET particles as compared to non-thermally treated. This implies that the alteration in surface morphology or loss of functional groups as a result of thermal degradation have reduced the potential toxicity of

cryomilled PET particles in terms of *Daphnia's* growth, survival, heart rate and reproduction. Among the three solvents used during the degradation process, Acetone was observed to be relatively more effective as indicated in the FTIR and NME analysis. This effect was further validated in the bioassay experiment where the Daphnia exhibited relatively higher growth measurements and lower mortality rate under the Acetone based PET exposure groups,

Future investigations are recommended to qualitatively access that what chemical changes or loss of functional groups in PET degradation have actually led to the reduced toxicity effects. Furthermore, it is imperative that forthcoming experiments evaluate more rigorous experimental conditions by increasing the treatment temperature and incorporating catalysts to determine whether a greater level of degradation can be achieved.

CHAPTER 5

DISCUSSION

5.1. Discussion

The present study investigated multiple aspects of microplastics journey in the environment. Generally, microplastic particles, either primary or secondary sources enter waste streams as well as freshwater bodies through different routes. Freshwater environments have not received as much attention as marine biota regarding the investigation of microplastics and their potential impact on living organisms. Hence, the current investigation sought to evaluate the environmental risk associated with microplastics in freshwater ecosystems. The impact of microplastics on the living organisms present in respective environment depends on many factors such as chemical nature and size of microplastics, exposure concentrations, presence of other pollutants such as heavy metals, organic pollutants, age of microplastics and duration of exposure (Wright et al.,2013). Thus, it was imperative to begin our analysis with the detection and identification of what types of microplastics could be present in the targeted freshwater samples. The experiment (chapter 2, section 2.2) used already established methods reported in literature for detection and identification of microplastics which includes optical microscopy (McDermid and McMullen, 2004, Costa et al., 2010, Norén, 2007, Collignon et al., 2012, Boerger et al., 2010, Lindborg et al., 2012, Heo et al., 2013), fluorescence microscopy (Stanton et al.,2019) and micro-Raman spectroscopy (Vianello et al., 2013, Browne et al., 2011, Ng and Obbard, 2006, Song et al., 2014). The results obtained from the identification

experiment (Chapter 2, section 2.3) indicated the presence of only one type microplastic 'Polyethylene terephthalate commonly known as PET' in the targeted water samples in very low quantity 0.01 ppm (only one sample out of five was detected positive for microplastics presence). This could be attributed to flushing of microplastics from surface water due to heavy rain, surface water run off and flooding (Hurley et al., 2018; Liu et al., 2019). The spectroscopy results were contrary to the results obtained from microscopic analysis. Many particles suspected as microplastics in microscopy analysis were identified as metallic in micro-Raman spectroscopic analysis. These findings are in agreement with Blair et al., 2019 who reported that non-MP pellets can be easily mistaken for microplastics by visual inspection alone.

Hence the present study suggests that micro-Raman spectroscopy is found to be most reliable analytical technique for qualitative as well as quantitative analysis of microplastics of varying size and shape in complex environmental samples.

Following the identification, the next objective was to perform a bioassay experiment to evaluate the potential toxicity of this microplastic type PET on freshwater model organism *Daphnia pulex*. Initially environmental samples were hoped to be used for subsequent bioassays but since the quantities of microplastics was detected were very low and complex nature of sample, laboratory prepared microplastic particles were prepared for controlled studies with defined amounts. Cyogenic milling of two common types of

microplastics i) Low density polyethylene LDPE and ii) Polyethylene terephthalate PET was performed and microplastics with broad size distribution (10-100 microns) with rough surface morphologies was obtained. It is known that these microplastics to adsorb other toxins, particularly heavy metals on their surface, thus combined toxicity analysis was also performed in parallel with the single exposure of microplastics and MP with heavy metals. The results indicated that selected microplastics had moderate toxicity effects on Daphnia's growth, reproduction and heart rate (chapter 3, section 3.3) whereas a significant effect on survival rate was observed at 1mg/l exposure concentration for seven days. These findings highlight variations in the literature, such as previous studies of Ziajahromi et al.,2017 who reported a significant decrease in daphnia body length after exposing them to PE microplastics compared to an increase in body length and width of daphnia under microplastic exposure (Rabus and Laforsch 2021). Hanees K, 2017 also reported an inconsistent and small change in daphnia body measurement in response to PS microplastics. Hence a contradictory information exist on altered morphological behaviour in response to microplastic exposure. These differences could be attributed to varying concentrations of PE used in previous experiments highlighting the need for a consistent ecotoxicological approach.

Reproduction was marginally ($p=0.05$) affected by all the treatment groups in the studied period of time. In seven days period only one brood was released

and none of the organisms produced a second brood. This agreed with Gersan An, 2024 who reported a reduced reproductive activity in response to PET microplastics. Mortality has been observed to be the most significant response of microplastic exposure in this study (100 % mortality at day 7 in three treatment groups) which agrees with many of the previous studies. Various previous studies have reported an increase in rate of mortality in response to different microplastic types (Jemec et al., 2016; Rehse et al., 2016; Frydkjær et al., 2017; Song et al., 2021). Naetal 2023 suggested that the chronic toxicity of microplastics induce oxidative stress that leads to high mortality rate. The findings are also consistent with Lei et al 2018 who reported reduced survival rate in response to PE microplastics. Seeing the sub-lethal effects of microplastics, RNA Seq analysis was performed. To date only limited studies have been conducted to evaluate the toxicity effects at molecular level. Most of the studies focused on specific biomarkers particularly for oxidative stress. A very recent study by Jiang et al 2023 reported toxicological effects of PET microplastics using RNA Seq analysis. The results from the experiment are in consistent with those of Jiang et al., 2023 who reported upregulated expression of superoxide dismutase. However, the other two antioxidant enzymes (CAT and Glutathione) reportedly showing overexpression were not observed in our results. Expression changes oxidative stress, pathway proteins observed in the results have never been reported previously. These include Apolipoproteins, vitellogenin's and Uncoupling

proteins UCPs. All these proteins play important role in antioxidant defence mechanism (Rainville et al., 2014, Mendez-Romero, 2020). Future investigations are needed to elucidate the mechanism underlying the interaction between these genes and oxidative stress pathway under varied conditions.

Lastly, microplastics in the environment undergo natural degradation process due to presence of various degrading factors in the environment such as microbes, UV light, high pressure, and temperature conditions. Hence, an analysis was performed to do microwave- assisted thermal degradation of PET microplastic in presence of three different solvents (HCl, Acetone and HCl). The objective of using different solvents was to see the effect of different pH conditions on the degradation of microplastics. Where only few studies exist that used other degradation methods such as UV light (Cao et al., 2022, Fadli et al., 2021), this study is the first one as per our knowledge, to report the effect of microwave assisted thermal treatment on PET and LDPE microplastic particle degradation. The study further investigated the post degradation behaviour for Fe and Pb metal adsorption, following a bioassay to see the impact of degraded PET particles on *Daphnia's* physiological responses. The results suggested that although the degradation at the present experiment condition was less, the partial degradation does exhibited a reduced adsorption potential for metals and subsequently reduced toxicity effects in terms of *Daphnia* growth, heartrate, reproduction

and survival.

5.2. Conclusion and future recommendations

Rivers are contaminated with microplastics but some of the analytical methods (such as microscopy) to detect them can overestimate the quantities. More sophisticated techniques, such as micro-Raman spectroscopy, although expensive, are better for precise qualitative as well as quantitative assessment for different kinds of environmental samples (as suggested in chapter 2).

LDPE and PET microplastics (10-100 microns) have tendency to adsorb other pollutants such as heavy metals (particularly Fe and Pb as reported in chapter 3) resulting in their bioaccumulation. However, this adsorption behaviour can be reduced because of thermal degradation of microplastic particles Concluded in chapter 4). Microplastics show sub lethal impact on invertebrates at physiological as well as molecular level at even very low concentrations (1mg/l investigated in bioassay experiments chapter 3 and 4). Although Sub-lethal effects of microplastics and metal adsorbed microplastics are observed in a model freshwater organism, however no consistent approach to the eco-toxicological assessment of microplastics has been taken in the literature and studies are needed to draw up guidelines to bioassay. According to a recent critical review by Ruijter et al.,2020, there exists an inadequate consistency and lack of standardized procedures for microplastics ecotoxic assessment. Most of the studies assessing microplastics

toxicity follows OECD guidelines for testing chemicals However, given that the toxicity of microplastics depends on numerous other factors, it is imperative to establish distinct guidelines that outline the specific experimental conditions.

Based on these findings, this work suggest the following recommendations:

1. Sampling of freshwater from sediment, flowing water, still water, at the point of effluent should be taken.
2. Efficient analytical techniques such as Micro-Raman should be used to investigate environmental exposure levels thoroughly.
3. Thermal treatment of wastewater reduces adsorption to PET, this study could be expanded to include a wider range of plastics and conditions and degradation treatments such as use of catalyst.
4. Ecotoxicological guidelines and standards need to be produced for freshwater organisms which should include standard use of reference materials, co-toxicants and more investigation parameters including molecular pathways .

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