INVESTIGATING THE TOXICOLOGICAL EFFECTS OF MICROPLASTICS EXPOSURE TO WATER FLEAS

(*Daphnia magna*)

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PP: Polypropylene

HDPE: High-Density Polyethylene

LDPE: Low-Density Polyethylene

LC50: Lethal Concentration 50%

mg/L: Milligrams per Liter

%: Percentage

µL: Microliters

cm: Centimeters

mL: Milliliters

mg: Milligrams

mm: Millimeters

Abstract

The ubiquitous presence of microplastics across all aquatic ecosystems, stemming from plastic pollution, necessitates their classification as emerging contaminants. This widespread distribution is concerning due to the propensity of aquatic organisms to ingest microplastics, this behavior likely arises from the similarity between microplastics and natural organic matter suspended in the water column. The current study investigated the toxicological impacts of microplastics of three prevalent plastic polymers—high-density polyethylene (HDPE), low-density polyethylene (LDPE), and polypropylene (PP) on the freshwater crustacean *Daphnia magna* in mono exposure and co-exposure settings. The microplastics ranging size 0-32 μm were synthesized in the laboratory in concentrations ranging from 30 to 150 mg/l. The lethal concentration 50 (LC50) for combined exposure was determined to be 77 mg/L, contrasting with LC50 values of 120 mg/L, 123 mg/L, and 109 mg/L for exposures to PP, LDPE, and HDPE alone, respectively. This variability in toxicity levels is attributed to the differential densities of the polymers and the distinct preferences of *Daphnia magna* for specific polymers within the aquatic environment. Co-exposure also induced a 10% increase in microplastic ingestion due to compromised egestion mechanisms, alongside a 21% reduction in reproductive rates due to impaired reproductive functionality under stress-induced conditions. Furthermore, combined exposure resulted in reduced population densities, with LDPE exhibiting the highest absorption rates at lower concentrations, followed by HDPE and PP at higher concentrations. Moreover, Daphnia exhibited extreme erratic swimming patterns indicative of heightened behavioral stress under combined exposure conditions relative to singular exposures. These findings underscore the potential threat that synergistic interactions between multiple microplastic polymers pose to *Daphnia magna* in realistic environmental scenarios.

CHAPTER 1

INTRODUCTION

Overview of microplastics

Microplastics, as the name describes, are tiny particles of plastic polymers that measure less than 5 micrometres in size (Bank & Hansson, 2022). They are ubiquitous in the marine environment and are found within the oceans, lakes, and all other environments (Horton & Dixon, 2018). The pollution caused by microplastics and plastic debris is one of the most emerging and pressing global issue. Microplastic particles are predominantly found in lake surfaces, sediments, soil but in some cases they are also present in the air around us (Jambeck et al., 2015).

Abundance of microplastics

Based on over 8200 microplastic samples from world oceans, soil, and terrestrial bodies. After precise modelling, it was calculated that there are almost 24 trillion pieces of microplastics present in the world's oceans, soil and terrestrial environment. These 24 trillion particles have a combined weight of 82,000 to 5,78,000 tons which is equal to 30 billion of 500 ml plastic bottles (Gaur et al., 2022). Almost 400 million tons of plastic waste is produced each year. These 400 million tonnes are projected to double by 2050 making it to 800 billion to be produced in 2051 (Priya et al., 2022). A good estimate of the current load of microplastics in all environments, it turns out to be around 5 billion tonnes. Even if microplastic production is stopped today, there will be microplastics that will exist in landfills, oceans, etc. The magnitude of plastics, particularly microplastics problem seems to be impossible to clean, collect or stop. Several scientists called this microplastic pollution a *Plastic time bomb*.

In 2019, a rough estimate states that around 3,70,000,000 tons of plastics was produced and after end of life, this 3,70,000,000 tons of plastic will eventually turn out to litter our ecosystems (Jambeck et al., 2015). In 2020, after the outbreak of corona virus the plastic production has increased, due to production of surgical masks, protective gears and other materials for protection against virus. Plastic polymers like polyacrylonitrile, polyethylene and polyethylene tetrathlalete were extensively used and produced. These plastic raw materials will eventually end up in the environment after the end of their life cycle (Anbumani & Kakkar, 2018).

Sources of microplastics in the environment

Microplastics presence has been reported from hand washes to cosmetic items to waste water treatment plant residues and their filters, microplastics are everywhere polluting our environment (Gasperi et al., 2018). Recent researches are carried out on microplastics and their synergistic effects on our oceans, lakes, soil and the impacts on marine and aquatic life. Researchers are working on this emerging pollutant and are trying to convince policymakers to prepare microplastic pollution abatement laws and policies and approve it from the governments to curb this microplastic pollution.

The main source of production of microplastics is anthropogenic activities because plastics are used in commercial and consumer goods which are bound to be disposed at the end of their life cycle and most of this disposed plastic will enter the environment at the end of its life cycle (Shim & Thomposon, 2015). Microplastics are unintentionally produced on an anthropogenic scale due to the wear and tear of plastic items, abrasion of the plastic products, and peeling and flicking off plastic items (Hidalgo-Ruz et al., 2012). Microplastic production results from the commercial manufacturing processes and textile industries, there are two sources of production of microplastics, either they are produced *directly or indirectly.* The direct production of microplastics is from industries such as chemical exfoliants, commercial plastic manufacturing or commercial packaging manufacturing etc (Andrady, 2011). The other source of microplastics are the indirect sources like commodity plastics which are physically chemically and biologically fragmented by anthropogenic or other activities to the plastic residues or debris which end up as microplastics (Carpenter et al., 1972).

Types of microplastics

Microplastics are of different types depending upon the polymer types these polymer types include high-density polyethylene, low density polyethylene polypropylene, polyethylene, PET, PVC, and all other plastic polymers which are used in the production of plastic items (Marturano et al., 2019). These microplastics can transform in the environment, this transformation is usually brought by chemical, physical or other biological processes. These transformations are also studied by various scientists and are considered to be dangerous for aquatic life (Issac & Kandasubramanian, 2021). Microplastics are released throughout the plastics value chain during transport, production, use, or at the end of life. Microplastics can be divided into two major types depending on the formation and the processes involved, which are primary and secondary microplastics.

Primary microplastics

Primary microplastics are the plastics that are directly produced and then released into the environment as plastic particles, they are pristine in nature. Primary microplastics are considered the main source of plastics in the environment as there are almost 3,000,000 tonnes of primary microplastic particles released annually according to European Environment Agency (*From Rivers to the Sea — the Pathways and the Outcome — European Environment Agency*, 2023.).

Secondary microplastics

Secondary microplastics, which are formed due to the breakdown of larger plastic items. They are typically produced due to the mismanagement of plastic waste which is subjected to weathering or aging. Secondary microplastics are also produced due to anthropogenic activities like when plastic products are discarded. Usually, secondary microplastics are often found in landfills, dump sites, or ocean beds (González-Fernández et al., 2021). Secondary microplastics are usually produced by synthetic textiles, almost 35% of microplastics released to the oceans originate from these textiles which makes them the number one pollutant of the environment in terms of microplastics. These microfibers released by textiles are the main source of ocean pollution in Europe (Meijer et al., 2021).

Degradation of microplastics

Currently, there are not many methods to degrade plastics, but in the environment, degradation occurs naturally and there are several processes involved in the degradation of microplastics. The microplastics in the environment naturally degrade by processes like, mechanically chemically or biologically. The degradation of microplastics depends on the characteristic of polymer, such as additive structure and chemical composition of the polymer as well as the environmental conditions to which the plastics are exposed such as temperature, their depositional matrices like air, water, soil and humidity and other environments, exposure to sunlight or they were buried beneath the water column or in the benthic layer, these factors decide the degradation potential of microplastics (Corcoran, 2022). The combination of these factors is responsible for microplastic degradation.

Pakistan's scenario

In Pakistan, around 200,000 tons of plastic waste is released into the ocean. This plastic waste usually contains secondary microplastics, which comes from coastal living areas and through industrial runoff products. River Indus is considered as the most polluted river in Pakistan. Almost 6000 to 8000 industries are operating in Pakistan who are responsible for producing around 1,000,000 tons of plastic waste in Pakistan. Out of these 1,000,000 tonnes of plastic 65% of these are plastics and they end up as trash in Pakistan. It is anticipated to rise 15% yearly. Out of this plastic waste, microplastics are generated through different processes like weathering, physical, chemical transformation of this plastic waste (*Plastic Pollution | WWF*, 2022). In Pakistan, there are not much studies showing the concentration of plastics in different environments in Pakistan but some studies shows that traces of microplastics were found in lakes of Pakistan such as Rawal lake in Islamabad having LDPE, HDPE PP, Polystyrene. In Rawal lake the concentration of plastic particles ranged from 6.4 to 8.8 particles per metre cube (Bashir & Hashmi, 2022). The microplastics were also found in surface water and sediments from kalarkahar lakes, different studies are showing that there are microplastics present in soil, in compost and Oceans particularly the Arabian Sea (T et al., 2020).

Effects of microplastics on human health

On the human level, there are several researches and theories which speculate that these plastics specs might be harmful to human beings but there is no or less proven research showing the effect of microplastics on the humans. The theories include thin fibres of asbestos or the foreign presence of microplastics, which can inflame the lungs and can lead to cancer (Guo et al., 2012). They can exhibit negative effects through the chemical toxicity because of presence of plasticizers stabilizers or pigments which are present in the plastics, they can eventually interfere with hormonal systems of human beings but there is still a gap that exists that how these microplastic particles travel throughout our bodies. Some recent articles published that there were specs of microplastics found in tissues of lungs, kidneys (Halden, 2010).

Effects of microplastics on marine organisms

The marine organisms, these are the organisms that swallow plastic particles which are of no nutritional value and and then accumulate in their bodies. Almost every marine organism be it copepods, shrimps, *Daphnia magna*, or larger organisms like sea turtles, fishes, octopuses etc consumes microplastics, when autopsied it was seen that there were microplastic particles in their cuts or tissues (Cole et al., 2013). Due to the presence of microplastics in the marine environment, the marine organisms are at the greatest risk of survival. When they encounter microplastics, they move slowly and reproduce less frequently and doesn't eat that much food which they require for their normal functioning. Hence the zooplanktons, which are the base of marine food web is at the greatest risk of microplastic pollution because impacts on zooplanktons eventually affects the fish stocks and the world population is on the brink of microplastics consumption due to this (Baulch & Perry, 2014).

Microplastics and food chain transfer

Marine organisms and aquatic organisms readily consume microplastics, as they confuse their food with these tiny plastic specks. In this way microplastics starts the journey in our food web from the very base of the trophic level which are zooplanktons. This is now a fact that microplastics are transferred through marine or aquatic food web but there are less models which are developed to investigate that how the biomagnification, bioaccumulation of the microplastics occur and how they travel through the food chain (Farrell & Nelson, 2013). There are studies which prove that the microplastics transfer across all 5 main trophic levels is happening, these study support the fact that by magnification of microplastics from lower to higher trophic level is occurring (Wang et al., 2019). Generally, lab studies haven't reported that much trophic transfer of microplastics due to longer experimental time constraints and availability of less data.

Overview and characteristics of Daphnia magna

Daphnia magna are infamously called as water fleas, *Daphnia magna* are planktonic crustaceans they belong to class branchiopod. *Daphnia magna* is a Cladocera, they are characterized by their flattened leaf like legs which produces water currents for the filtering operation due to this quality they are also called as filter feeders. *Daphnia magma*'s English name, water flea, comes from the jumping-like action they exhibit while swimming. This behaviour is caused by the beating of the huge antennae, which they use to navigate through the water. The rapid downbeat causes a speedy upward movement, whereas the creatures comparatively high density causes sinking. *Daphnia magna* descend to the ground quickly when they are still (Hobaek & Larsson, 1990).

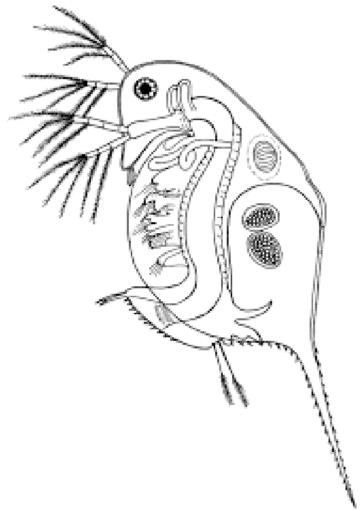


Figure 1: *Daphnia magna*

These water fleas are small in size, they are around 2-5 micrometre long and they exhibit shape of a kidney bean, their body is transparent and a shell-like structure which is called carapace. The body is usually made of chitin for the filtering apparatus, it has thoracic limbs and ventral openings which help *Daphnia magna* to move and filter the water, it has a hook shaped intestine with two digestive ceca (Mittmann et al., 2014).

The main food of *Daphnia magna* is filtering the suspended particles present in the water. Their thoracic appendages which generates water current within the opening of the carapace, which permits the collection and ingestion of unicellular algae, detritus, bacteria and anything which is suspended in water .Their filtration rate depends on temperature, body size, quality of food, concentration of oxygen and the pH of water the primary diet of *Daphnia magna* is zooplanktons and phytoplankton's, they can also consume fungal spores (Bekker et al., 2018).

In *Daphnia magna* males are smaller than females like males are only two mm long but females are three to five mm long and have longer attenuates and different appendages which help them during the mating process. *Daphnia magna* are found in almost every water body ranging from huge lakes to small temporary ponds to habitats or ponds rich in organic matter. They are the main zooplankton which is dominant in ponds and lakes and they make the base of the food web in ponds. In many lakes *Daphnia magna* are food for fishes. They are also found in brackish waters with salinity up to 8 parts per trillion and likes to survive between 18 to 22 degrees of temperature but they can tolerate much broader range of temperature (Yin et al., 2023).

The *Daphnia magna* can reproduce sexually and asexually and have a cyclic parthenogenic life cycle. When they are producing asexually females produced eggs that develop into the clones, this usually happens during adverse conditions when there is no food available the high temperature or low temperature and the population density is very high. When favourable condition returns this species produced sexually during this process female produce eggs which are fertilized by males and then the eggs are encased in this ephippia, these eggs remain in the female bag and then when favourable conditions are present female reproduces and late months these eggs usually burst and offspring is born. Their production of offspring is on peak during the spring season but exact usually produced during summer season and winters the females can produce egg after every four days periods and they remain in their brood chamber for like two to three days. A female produces around 25 time during their life her life cycle but the average is considered to be six times only. Typical life cycle of *Daphnia magna* is from 1 to 56 days but taking average becomes around 40 days a definite survives naturally (Hobaek & Larsson, 1990).

Role of Daphnia magna magna in the aquatic food web and water quality

Daphnia magna play a key part in aquatic food web as they are the prey to fishes and other invertebrates, *Daphnia magna* are host to number of fungi, bacteria, amoeba, tapeworm and nematodes, they also have commensal and parasitic rule in the ecosystem and are indicators of cleaner water in lakes pond because they usually eat algae and other detritus which is in the water (Pan et al., 2022).

Daphnia magna are considered as an indicator for water quality and they are used in water toxicity tests for detection of pollutants in water *Daphnia magna* are easily cultivated are commonly fed to fishes. *Daphnia magna* usually migrates towards the upper level of water body during night time and then come back to the lower level in early morning or daytime. This is their main predator avoiding strategy like, during the daytime they hide from fish and go to deeper depths and when night time comes take advantage of food and come towards this surface for feeding like algae and other substances. This motion helps them to survive in an ecosystem in which they are living we can call this behaviour of *Daphnia magna* as phototaxis. Currently the population of *Daphnia magna* has increased resistance towards pesticides and salinity and other environmental toxins they can easily survive now in any kind of water and environment (Koelmans et al., 2013).

Use of Daphnia magna magna as test organism

Daphnia magna is the main experimental Organism in most of the ecotoxicity studies, there are many reasons for choosing *Daphnia magna* as model organisms in the current study like, due to the presence of transparent body and easily visible anatomical structures under the microscope it is easy to have *Daphnia magna* assessed for microplastic based studies. The culture maintenance of *Daphnia magna* in Pakistan is not a big issue it replicates from hundreds to thousands in a matter of days, the main advantage it gives us is first generation time is quick, limited space requirement and feeding and maintenance is very simple and cheap

The *Daphnia magna* is also a main Organism in all OECD guidelines for testing of chemicals like their immobilization tests, their reproduction test, their acute toxicity tests by exposing them to different toxins in the environment so it is very easy to process in lab (*Test No. 202: Daphnia Sp. Acute Immobilisation Test*, 2004). Their organs can be well seen under a simple microscope and effect of microplastics can easily be known by this thing.

In the current study *Daphnia magna* was used due to its easy availability in Pakistan, it's lower reproduction time, its resistance towards the climate and food and due to its size. The interactions of *Daphnia magna* with microplastics were to analyse and the literature supports the fact that *Daphnia magna* provided some great responses towards microplastic studies so that's why *Daphnia magna* was chosen to be the main organism for this study

There are several studies which have reported the presence of microplastics in *Daphnia magna* of different types and different polymers and shapes even the reproduction rates their multi-generational effects were also seen by the researchers. Microplastics have been the most abundant pollutant in the oceans and water bodies were exposed to the most abundant organisms present in the oceans and water bodies so the interaction of both was seen and how microplastic impacts *Daphnia magna* life cycle was observed. *Daphnia magna* usually confuses the algae with microplastics and eventually, it takes or filters the microplastics in its gut which are of no nutritional value these microplastics remain inside *Daphnia magna* indefinitely, the fishes eat them because *Daphnia magna* resides at the main base of the trophic level and in this way the microplastics start inculcating in the marine food web. This matter is of grave concern nowadays, many researchers have approved the trophic transfer of microplastics and some are finding ways to prove that microplastics end up in our white meat, etc.

Objectives of the study

The following were the objectives of this study;

- To prepare different types (PP, LDPE, HDPE) of microplastic suspensions on a laboratory scale
- To determine the toxicological effects of single microplastics type on *Daphnia magna*.
- To determine the toxicological effects of co-exposure of different microplastics on *Daphnia magna*.

CHAPTER 2

LITERATURE REVIEW

Microplastics pose a significant and emerging threat to the environment, attracting considerable attention from researchers. These scholars are engaged in studies focusing on the toxicity, characteristics, potential harms, and threats associated with microplastics, as well as ecotoxicological investigations pertaining to these minute plastic particles. Various research endeavors have been undertaken to investigate the impacts of microplastics, including their trophic transfer and effects on *Daphnia magna*.

2.1 Microplastics in ecosystems

Amelia et al. (2021) conducted a review highlighting microplastics as one of the primary pollutants in oceanic and marine ecosystems. The study primarily aimed to examine the potential of microorganisms as vectors for other hazardous pollutants. The literature review revealed that the characteristics, chemical interactions, and properties of microplastics significantly influence the sorption of environmental pollutants onto them. The dominant microplastic types found in the oceans are typically fibers or fragments originating from anthropogenic activities. These microplastics possess the potential to transport contaminants such as BPA, PAH, hydrophobic compounds, and heavy metals from the environment. It was concluded that pollutants can sorb to microplastics, entering the food web. The adverse effects of microplastics become more pronounced when they interact with environmental contaminants, surpassing the risks posed by microplastics alone.

Microplastics are ubiquitously present, but their entry into the oceans occurs through rivers and other bodies of water that eventually reach the sea. Kataoka et al. (2019) investigated the sources and inflow of microplastics in various river environments in Japan. Microplastic samples were collected from multiple locations along Japanese rivers and subjected to physicochemical analysis. Stainless steel bottles were used to transport the collected samples to the laboratory. Microplastic concentrations were evaluated in the lab using a Stereoscopic Microscope, Fourier Transform Infrared Spectroscopy (FT-IR), and other physicochemical parameters such as BOD, COD, pH, and dissolved oxygen levels. The results demonstrated the presence of microplastics in all the samples. Seasonal comparisons indicated approximately 3.5 times higher microplastic concentrations from October to March compared to the period from April to September, suggesting possible seasonal variations. Moreover, the concentration of microplastics increased in correlation with higher BOD and dissolved oxygen levels in the water. The study concluded that microplastics from rivers ultimately reach the ocean, and events like flash floods can transport them into the soil. It emphasized the urgent need to assess the inflow of microplastics from rivers to oceans, ensuring easily accessible data on microplastic concentrations that are continuously monitored throughout all seasons.

The presence of microplastics has been documented in surface waters across different regions worldwide. Li et al. (2020) assessed the concentration of microplastics in surface water and sediments of the Yangtze River estuary in China. The Yangtze River is China's largest river, accounting for the world's highest discharge volume. Although the presence of microplastics in the Yangtze estuary had been reported in the literature, limited information was available regarding the types, sizes, and shapes of the plastic particles found. Samples were collected from the estuary and analyzed in the laboratory using a stereomicroscope. The number of particles in the surface water and sediments was quantified, and FT-IR analysis was performed to determine the nature of the plastic particles. The study revealed an abundance of microplastics in the surface water of the Yangtze estuary, ranging from 0 to 259 microplastic items per cubic meter. In sediments, the concentration ranged from 10 to 60 microplastic particles per kilogram. The researchers suggested the implementation of microplastic monitoring at all river inflow points to obtain comprehensive details about microplastic

2.2 Microplastics presence in Pakistan

In Pakistan, monitoring of microplastics is not a common focus among researchers. However, a recent study conducted by Bashir & Hashmi (2022) investigated the sources of microplastics in the surface water of Rawal Lake and estimated their abundance. Understanding the inflow process and water sources is crucial for assessing microplastic concentrations. The researchers selected six tributaries of Rawal Lake as sampling sites and employed a self-built trawl to confirm the presence of microplastics. The researchers hypothetically identified the influx points and confirmed the presence of microplastics using the wet sieving method with various sieves. FT-IR analysis was conducted to identify the nature and polymer composition of the microplastics, and fluorescence microscopy was used for imaging purposes. The study concluded that the concentration of microplastics in Rawal Lake ranged from 6 to 8 microplastic particles per cubic meter, with polypropylene (PP) being the dominant type. Additionally, the majority of microplastics measured range between 0.1-0.3 micrometres in size, and deep transparent films were the predominant form of plastic observed in Rawal Lake.

2.3 Microplastics availability

Microplastics are commercially available and in various types of shapes and almost every polymer of microplastic is available for the synthetic lab-based experiments but the cost of microplastics which commercially available is very high it ranges from around 100 to 600$ for a 5ml suspension, which is fluorescently labelled. There are different methods to prepare microplastics on a lab scale and then confirming their purity and polymer by different methods. Chialenza (Rodríguez Chialanza et al., 2018) in his study identified and quantified semi crystalline microplastics by image analysis and differential scanning calorimetry. In this research the available microplastics were selected according to environmental prevalence of this plastics and their semi crystalline material. The manual grinding of these pellets with silicon carbide paper resulted in production of microplastic debris or powder which was then seived for desired range and then the image analysis is differential scanning calorimetry. This made way for a new method of identifying and producing microplastics in the environmental sample with the help of differential scanning calorimetry which identified the chemical and performed mass quantification of particles. It was concluded that high density polyethylene has the best identification and quantification capability in terms of thermal conductivity, this gives a new method or an technique to identify and Mass quantify the particles based on temperature and heat flow properties.

Microplastics are presence has been reported in almost every freshwater body, be it ocean, lakes, rivers, ponds etc . Microplastics in these freshwater bodies interfere with the zooplanktons present in the waterbodies. The zooplanktons consume microplastics as microplastics mimic the behaviour of algae or other food source. Zara botterell and her colleagues (Botterell et al., 2022) studied the ingestion of microplastics in this zooplanktons from the from strait of the Arctic. The main objective of the research was to investigate ingestion of microplastic zooplankton's present in Artic zone. The method used to identify microplastics was polymer identification approach. It was seen that microplastics were present in all the types of zooplanktons, all zooplanktons were enzymatically digested and then filtration of these samples were done with the help of 13 mm sieve and particles were subjected to the FT-IR analysis, the quantification was performed with the help of a software. It was seen that all the types of zooplanktons consumed microplastics in 0-50mm range. The amphipods had ingested more plastics than copepods. It was concluded that the microplastics are bioavailable to the zooplanktons in the oceans and freshwater bodies. These species are of most importance as they form the food web and are at the most risk of microplastics ingestion.

2.4 Microplastics and Daphnia magna

Canniff and Hoang (2018) studied the ingestion of microplastics by *Daphnia magna* and role of algal growth in the ingestion of microplastics. In the experimental setup the *Daphnia magna* were exposed to different types of microplastics and algal solutions for different period of time and their biological characteristics were monitored. From 7 to 21 days, the parameters were noted down and the ingestion was confirmed with the help of acid digestion of organism and then quantifying the microplastics under a stereomicroscope. It was observed that the *Daphnia magna* has ingested microplastics in the ranges of 63-75 microns and growth of algae was also enhanced by the microplastics and were uptaken by *Daphnia magna*. It was concluded that microplastics have effects on biological parameters of *Daphnia magna*.

An extensive research was conducted by Imhof and co-researchers. on the topic of effects of microplastics on *Daphnia magna's* morphological life history and molecular levels (Imhof et al., 2017). In this study the effects of two polymers of microplastics on the morphology and life history of *Daphnia magna* was observed. The *Daphnia magna* were exposed to the microplastics mixtures for 48 hours. The genes expression and changes of *Daphnia magna* after exposure to microplastics were quantified using PCR analysis and all other parameters were noted down and the relationships were calculated statistically. It was concluded that microplastic particles of less than 40 micrometre were present inside the gut of *Daphnia magna* and an average of 30 particles were present within the digestive tract. There were less significant relations found for morphological parameters or reproductive parameters, but there were alteration in gene expression observed after exposure for 48 hours.

Gerdes and researchers (Gerdes et al., 2019) developed a novel method to assess the effect of microplastics in a suspension. Due to less data on ecotoxicological methods on microplastics, the research was carried out to assess the microplastic effects on organisms a novel approach using serial dilution of microplastics and reference particles like clay and cellulose was used. The organism used to view the effect of these microplastics was *Daphnia magna*. The Organism was exposed to different concentrations of PET and immobilization tests were carried out along with little concentration for the plastic mixtures. The organisms were exposed to 48 hours and according to the protocols of OECD, the acute immobilisation test of *Daphnia magna* was performed with some modifications. It was concluded that the over 50 milligram per litre of microplastics in a solution caused deaths of 50% of population of *Daphnia magna* present in the solution. The maximum lethal effects to *Daphnia magna* were seen then the exposure time was increased from 48 to 96 hours, this caused starvation in *Daphnia magna* and their energy reserves were depleted because of this and they were immobile and energy-less. Microplastics caused many stresses to the organism and it is evident from the results. This research helped in producing a potential standard for ecotoxicological testing of some particles like microplastics on the organisms like *Daphnia magna*.

2.5 Solutions to microplastics pollution

Bioremediation of microplastics by different organisms a coming of age topic and researchers have started to work on this novel approach to get rid of microplastics. In the chapter published by Ghaznvini (Hadian-Ghazvini et al., 2022) Showed that how the mismanagement of plastic waste is entering the ocean and producing marine debris with the current estimates it is expected that there would be more plastic items of macro size in the ocean then the fishes. Plastic does not decompose naturally, it takes a long time to decompose. Bioremediation and degradation are two approaches which are fancy, but hard to do approaches. Biodegradation involves enzymatic engineering which takes into account some different chemical, physical molecular, crystallinity and other features of the microplastics and then the decomposition occurs. The natural degradation of microplastics is done by a sunlight, heat decreases the surface hydrophobicity of microplastics and in this way the microorganisms form biofilms on the surface of microplastics and then the Organism biodegrades the microplastics. The adsorption of antibiotics on microplastics concern because it is producing antibiotic resistant bacteria and genes which already concerning. Assimilation and mineralization of the tiny specks of plastics are last degradation steps. Some bacteria and species are key players in the biodegradation of microplastics, as they convert them into carbon dioxide to water. More research and insights are needed for the proper mechanisms of degradation of microplastics using the approach of bioremediation and biodegradation.

A review published by Aliko focused on getting rid of the marine pollution and use of bioremediation as an innovative attractive and a successful cleaning strategy (Aliko et al., 2022). Bioremediation is a new but, biologically and ecologically doable approach that enhances the ability of microorganisms to transform waste and toxic substances from one form to another. The microorganisms are playing a key role they detoxify our aquatic system in the coming years. The pathway which a microorganism follows like *rhodobacter marinum* or *pseudomonas*, *rhodococcous, streptomyces, enterobacter* is the typical path for which a microorganism takes. It involves four steps which is biodeterioration, biofragmentation then assimilation and mineralization. In the first step of bio deterioration there is buffering formation followed by a bio fragmentation in which there is secretion of enzymes by microorganisms followed by assimilation process which helps in the diffusion of the oligomer from the surface of microorganisms and then comes the last step which is mineralization in which the bacteria breaks down the plastic into secondary metabolites such as Carbondioxide water etc. The interest of scientists in biodegradation of microplastics is increasing use of microorganisms for this purpose is very innovative and a less harmful technique which poses no significant problem to the environment and may actually help the ecology. The innovation brought by the bioremediation approach ensures that the contaminants like microplastics or other harmful contaminants adsorbed to the microplastics are degraded using the microorganisms in the nature and when these species introduced to different ecosystems will eventually help and tackling problem of microplastic pollution.

CHAPTER 3

METHODOLOGY

The study was designed into four phases, first phase was to prepare microplastics indigenously, second phase was to rear the culture of *Daphnia magna* and Subsequent phase was to expose *Daphnia magna* to microplastics and last step was to carry out toxicological analysis. The methodologies for all the phases are described below in detail.

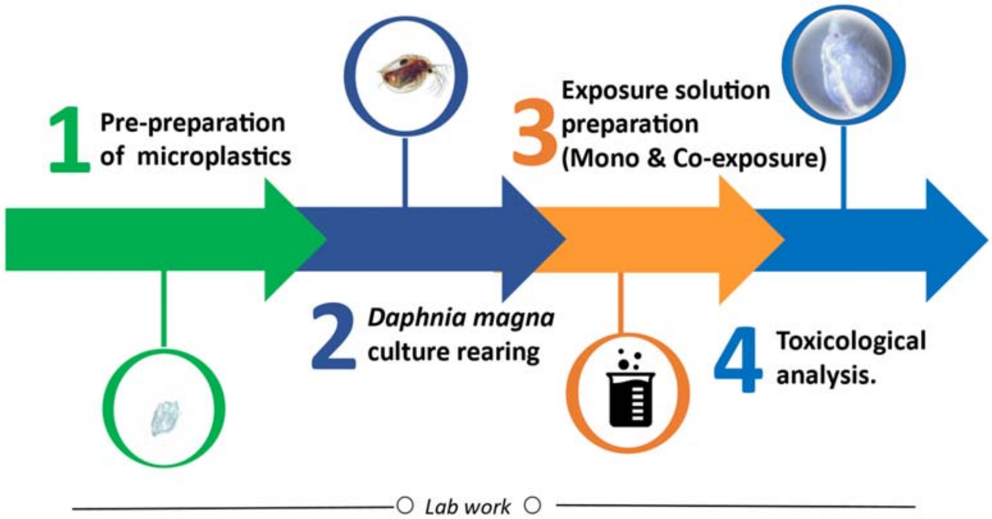


Figure 2: Phases of Experiment

3.1 Pre-preparation of microplastic suspensions

Plastics are man-made organic polymers that are formed by polymerization process (Thompson et al., 2004). One of the emerging forms of plastic pollution that has caught eye in recent years is microplastic pollution. The plastic beads were obtained from local vendor, which are used for injection molding process for plastic products like shopping bags, containers, bottles etc. The plastic beads were of three different types of polymers, PP, LDPE, HDPE. The beads were in transparent color and were in crystalline form. The beads were trademarked under Marlax, which is a standard used for the plastic manufacturing (*Marlex Polyolefin Plastics - Phantom Plastics*.). The plastics beads approximately 20g were collected in a petri dish and were washed with 95% pure ethanol and dried at room temperature to remove any impurity from the beads (Kim & Cho, 2020).

The dried clean plastic beads were placed in a glass petri dish for heating at its melting point, 50% of the temperature on which a polymer melts was maintained by the hotplate, this helped in producing pure and non-oxidized pallets of plastics.

- For PP, the beads were heated at 80 °C for 5 minutes
- For HDPE, melts at 65 °C for around 5 minutes
- For LDPE, melts at 60 °C for around 5 minutes

This process melts the plastic beads together and a shape or pallet which is ergonomically feasible to crush, is prepared out of the plastic beads.scha

Following the cooling of the plastic pallets, the subsequent step involved their grinding. A unique approach was employed, deviating from the conventional utilization of silicon carbide paper as documented by Rodríguez Chialanza et al. (2018). Instead, stainless steel nail filers were utilized to finely grind the plastic pallets, facilitating their conversion into irregularly sized plastic debris, each fragment measuring less than 5 microns. To ensure the integrity of the crushing process, stringent parameters were maintained to prevent external impurities from contaminating the samples. Grinding operations were conducted within a closed environment using equipment constructed from stainless steel and glass, thereby excluding any plastic components. Subsequently, the plastic debris underwent wet filtration to isolate microplastic fragments within the desired size range. ASTM standard sieves, specifically Sieve number 625 (20 microns) and Sieve number 450 (32 microns), manufactured by Endecotts, were employed for this purpose. The plastic debris was suspended in either distilled water or acetone, supplemented with a surfactant, Sodium Dodecyl Sulphate (SDS), using a microspatula. SDS served to diminish surface tension and aggregation, ensuring uniform submergence of microplastic fragments in the liquid medium (Jiang et al., 2021).

The filtration assembly, incorporating the designated sieves, facilitated the separation of microplastics from the suspension. The filtrate yielded a homogeneous solution of microplastics in water. Subsequently, the microplastic fragments were collected on 0.45-micron filter paper and subjected to thorough washing with ethanol and hard water over a 24-hour period to eliminate any residual impurities (Kim & Cho, 2020).

The resultant microplastic fragments were then subjected to analysis using a Microscope and Particle Size Analyzer, followed by FT-IR Analysis to elucidate their chemical composition and structural characteristics.

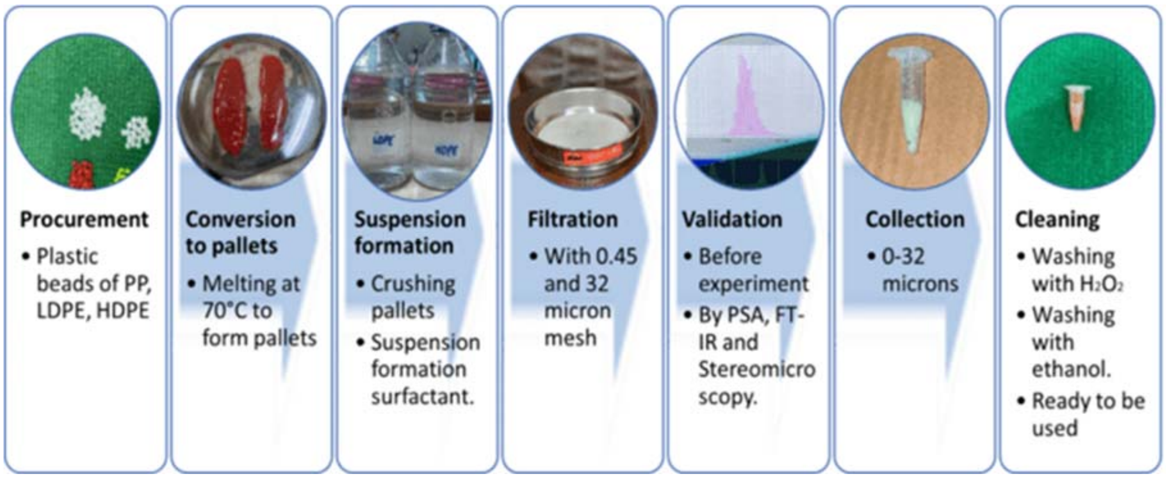


Figure 3: Steps followed for microplastics preperation

Size validation Particle Size Analyzer

A novel method of validating the sizes of microplastics used in the process, Horiba LA-300, Particle Size Analyzer with range of 0.1 to 600 microns was used, (Togawa & Kurozumi, 1999) the wet solution containing the surfactant and the microplastics was subjected to particle size analysis with prior sonication and circulation and the results were recorded. This method was adopted to quantify and calculate the sizes of plastic particles present. PSA works on the principle of Dynamic light scattering (DLS) and researchers have found a link of quantifying Microplastics by DLS (Wang et al., 2021). Before the results were calculated, the sample chamber was rinsed completely and sample after pouring was doubled to remove any bubbles present. A blank test was run to align the laser and results were recorded and the mean, mode and median of all the samples were calculated.

Figure 4: Particle Size Analyzer (Horiba LA 300)

Chemical characterization by 'Fourier-transform infrared' spectroscopy.

In order to validate the final product from the process i.e. microplastics, agilent technologies FT-IR (Model; Cary 630) was used in Attenuated Total Reflectance (ATR) mode in order to acquire the compositional information of the plastics particles which were analyzed (Veerasingam et al., 2020). The ATR technique allowed the microplastic particles to be directly observed in the powered form without any preparation (Xu et al., 2019).

The samples of PP, LDPE, and HDPE were subjected to FTIR spectroscopy.

Figure 5: FT-IR Spectroscope (Agilent Technologies)

Image recording by Stereomicroscope

In order to capture 3 dimensional pictures and length and width analysis of microplastics, a digital stereomicroscope was used (Model: Olympus DSX1000). The samples were observed in dried form for their imaging and the results were analysed with the help of software (DSX 1000).

Figure 6: Stereomicroscope (Olympus DSX-1000)

3.2 Daphnia magna culture preparation

The culture of *Daphnia magna* was obtained from a local aquariums shop, *Daphnia magna* in Pakistan is usually given to fishes as a high protein diet. The culture of *Daphnia magna* in a 100 ml glass jar was subjected to the Environmental Toxicology Laboratory in Institute of Environmental Sciences And Engineering (IESE) where the *Daphnia magna* were mass cultured for the experiment.

For the preparation of mass culture medium, three 2 Liter glass beakers were used and pure ground water from 400 feet depth was used as main source of water. The reason for using ground water as culture medium was to ensure there is no presence of residual chlorine as *Daphnia magna* are extremely sensitive to the chlorine (Polhill et al., 2022). The pH, Dissolved Oxygen and temperature were within the range in which *Daphnia magna* survives, detailed in Table 1. All these parameters were pre-tested using available equipment for pH, DO and temperature, chlorine. Microbial contamination was also monitored which was not present.

After making the water suitable for the production of *Daphnia magna*, the organisms from the glass jar were added to the three beakers and were subjected to mass culture. The light to dark cycle was set to 16:8 hours, light was provided by artificial as well as natural sunlight. After every 2 days the water was added to improve aeration as the DO levels must be above 3.5 mg/L. (*6.1. Daphnia and Moina*)

Table 1: Parameters for *Daphnia magna* culture rearing

Parameter	**Range**
pH	7-8
Dissolved oxygen	6-8 mg/L
Temperature	18-22 degree Celsius
Residual chlorine	0 ppm
Light/Dark cycle	16:8 hours

Figure 7: Culture rearing setup

The *Daphnia magna* culture was reared for 30 days before the start of experiment and the food provided to *Daphnia magna* was Bakers yeast, basically the instant yeast powder which is readily available from local shops, as *Saccharomyces cerevisiae.* The yeast was fed after 4-5 days and the dose was about 2ml/litre as yeast was given to the *Daphnia magna* mixture in liquid form to dissolve in water. Due to high fertility and less space available in the jars, *Daphnia magna* which were dead due to space constraints were discarded out and disposed safely. If the reproduction of *Daphnia magna* exceeded the capacity of beakers, 100+ *Daphnia magna* were taken, the beakers were washed and then a new culture was prepared by the previous method.

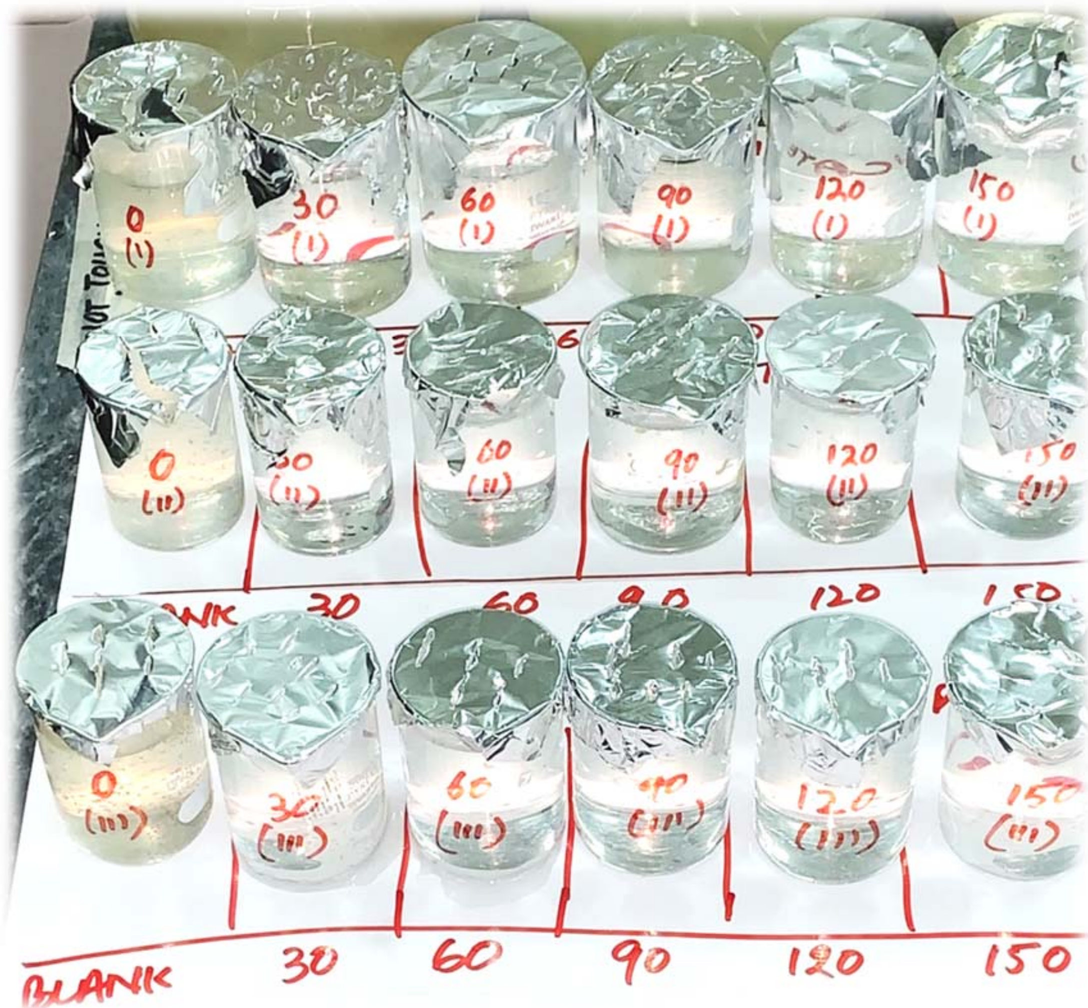


Figure 8: Experimental exposure setup, experiment performed in triplicates.

3.3 Exposure experiment

The experiment was designed in order to study the effects of ***mono exposure*** of microplastics and ***combined exposure*** of microplastic on different parameters of *Daphnia magna*. (Table 2)

Table 2: Exposure types of microplastics to *Daphnia magna*

Exposure Type	No of Daphnia	Exposure time	Exposure chamber	Exposure medium
Mono exposure PP	20	5	80 ml glass beakers	80 ml MPs water in 30, 60, 90, 120, 150 mg/L conc.
Mono exposure LDPE	20	5	80 ml glass Beakers	80 ml MPs water in 30, 60, 90, 120, 150 mg/L conc.
Mono exposure HDPE	20	5	80 ml Glass Beakers	80 ml MPs water in 30, 60, 90, 120, 150 mg/L conc.
Co-exposure	20	5	80 ml Glass Beakers	80 ml MPs water in 30, 60, 90, 120, 150 mg/L conc.

3.4 Toxicological analysis

There were five toxicological parameters to be studied, detailed methodology with the parameter is described below:

1. **Survival of *Daphnia magna***

To determine *survival of Daphnia magna*, which is in form of LC50, *Daphnia magna* from the culture medium were taken out and placed in a 2l glass beaker containing deionized water, the *Daphnia magna* were made to starve for at least 4 hours prior to the exposure to microplastics, because of this step the *Daphnia magna* would consume microplastics as soon as possible and microplastics usually mimic the behavior of food (Canniff & Hoang, 2018). *Daphnia magna* after starvation were exposed to solutions containing microplastics, 20 adult *Daphnia magna* were placed in 80 ml beakers with 50 ml of deionized water containing microplastics, the microplastics concentration for each type of microplastics in the mono -exposure experiment was 30, 60, 90, 120 and 150mg/l with a blank for each microplastic type. The microplastic fragments of PP, HDPE, LDPE were weighed and added to 500ml of deionized water and sonicated in order to remove any agglomerates, while pouring the solution into the 80ml beakers the stock suspension was agitated so that the microplastics are in a consistent number. For co-exposure mixture 33% of each microplastic polymer (PP, HDPE, LDPE) was mixed to form solutions of 30, 60, 90, 120 and 150 mg/L (Table 3). The exposure effects add to the novelty of the research as it depicts the natural environment in which all microplastic types are present and mostly in fragmented form. To calculate the survival of *Daphnia magna* in both of the exposures, 48-hour acute toxicity test was performed and 0.1 mg of feed was provided to the organisms in each exposure unit *Test No. 202: Daphnia Sp. Acute Immobilisation Test*, 2004. The observations were taken on the basis of death count or complete immobility, LC50 which is the indicator of survival, was calculated using the Probit Analysis.

Table 3: Microplastic stock solution preparation for all polymers

Plastic polymer	**MPs stock solution**					
	0	***30***	***60***	***90***	***120***	***150***
Polypropylene	300 ml exposure medium	30mg MPs in 1L non-chlorinated ground water	60 mg MPs in 1L non-chlorinated ground water	90 mg MPs in 1L non-chlorinated ground water	120 mg MPs in 1L non-chlorinated ground water	150 mg MPs in 1L non-chlorinated ground water
LDPE	300 ml exposure medium	30mg MPs in 1L non-chlorinated ground water	60 mg MPs in 1L non-chlorinated ground water	90 mg MPs in 1L non-chlorinated ground water	20 mg MPs in 1L non-chlorinated ground water	150 mg MPs in 1L non-chlorinated ground water
HDPE	300 ml exposure medium	30mg MPs in 1L non-chlorinated ground water	60 mg MPs in 1L non-chlorinated ground water	90 mg MPs in 1L non-chlorinated ground water	20 mg MPs in 1L non-chlorinated ground water	150 mg MPs in 1L non-chlorinated ground water
Co-exposure	300 ml exposure medium	30mg MPs in 1L non-chlorinated ground water	60 mg MPs in 1L non-chlorinated ground water	90 mg MPs in 1L non-chlorinated ground water	20 mg MPs in 1L non-chlorinated ground water	150 mg MPs in 1L non-chlorinated ground water

2. Ingestion and bioaccumulation

To study *ingestion and bioaccumulation* of microplastics into the *Daphnia magna*, organisms were exposed at similar concentrations to mono and co exposure microplastics, the microplastics were dyed with Nile red fluorescent dye in order to view the ingestion of microplastics into the *Daphnia magna*. 0.1 mg/10ml dye was prepared using acetone and plastics were added into the dye, filtered and washed thoroughly to remove any access dye, the plastics were then used to make suspensions of 30-150 mg/L. 20 *Daphnia magna* were exposed to fluorescently labelled microplastics of mono and co exposure for a period of 110 hours/5-days and were analyzed through a fluorescent microscope, model Optika B-350. To calculate the bioaccumulation of microplastics of both exposures, 40% i.e. Eight specimens of *Daphnia magna* were collected from each exposure beaker for subsequent analysis. These specimens underwent digestion using a modified draft method, where 50% nitric acid was employed for a duration of 3 hours at a temperature of 50 degrees Celsius. This digestion process was conducted for both exposure scenarios. The resulting digested solution was then diluted with deionized water and filtered through glass fiber filter paper, followed by air drying at room temperature. Notably, this digestion procedure adhered to the method outlined by Canniff (Canniff & Hoang, 2018), with certain modifications.

For the single exposures, the filter papers containing the digested samples were examined under a microscope to quantify the microplastics, with the number of particles per organism subsequently determined. Conversely, in the case of co-exposures, while microplastics were observed, their specific composition remained uncertain. To address this, the digested samples were filtered, and the filter paper harboring microplastics underwent ATR-FTIR analysis. This analytical technique facilitated the identification of predominant plastic types accumulated within *Daphnia magna* across varying concentrations. (Gerdes et al., 2019).

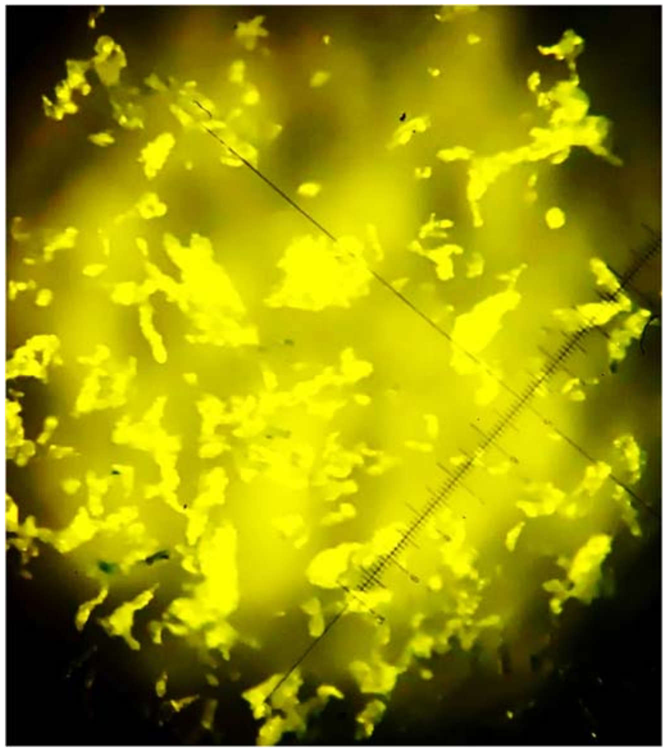


Figure 9: Fluorescently labelled microplastic in faeces of *Daphnia magna*.

3. Reproduction rate

A treatment replicate's *reproduction rate* per organism per day was computed by dividing the total number of neonates produced in a treatment replicate by a factor of days by the number of adult *Daphnia magna* residing in the same treatment replicate on the same day. The average 5-day cumulative number of neonates generated by a surviving adult, as well as the average reproductive rate each day for each treatment, were calculated and compared to observe if there was a significant difference between treatments (Imhof et al., 2017).

4. Population density

Population density was determined through two distinct methods. Initially, the density was calculated by enumerating the number of *Daphnia magna* present within a specified volume of water. Specifically, 10 milliliters (ml) of water was extracted from each replicate, and the total number of *Daphnia magna* within this sample was counted. The population density was then calculated by dividing the total count of *Daphnia magna* by the volume of water sampled.

Additionally, population density was assessed using a colony counter. In this method, the replicate water sample was poured into a petri dish, and the density of *Daphnia magna* colonies was observed visually. This technique provided a direct visual estimation of the population density. Both methods were employed to ensure comprehensive and accurate assessment of *Daphnia magna* population density, as outlined by Guilhermino et al. (2021).

5. Swimming behavior

To elucidate the swimming behavior of *Daphnia magna*, 1-minute-long videos of each replicate from both exposure types were recorded and subsequently subjected to swimming pattern analysis. Various swimming patterns, including hopping and sinking, cruising, and vertical swimming, were visually identified and noted, following the methodology outlined by Guilhermino et al. (2021).

To discern potential changes in swimming behavior indicative of stress in *Daphnia magna*, the recorded videos were analyzed using an insect tracking software called AnimApp. While typically available for both PC and Android platforms, for this study, the mobile application version of AnimApp was utilized. The software allowed for precise tracking of organism movement, with the threshold adjusted to detect *Daphnia magna* specifically. Subsequently, swimming trajectories were tracked and plotted to facilitate a comprehensive analysis of swimming behavior patterns, as per the approach described by Rao et al. (2019).

CHAPTER 4

RESULTS AND DISCUSSION

Crushing and sieving the plastics through wet filtration method resulted in the production of microplastic fragments, irregular in size. Plastic suspensions were prepared for FT-IR spectroscopy & particle size analysis. The prepared microplastics were used for exposure experiment in different concentrations.

4.1 Microplastics preparation

4.1.1 Sieving and stereomicroscopy

According to Rodríguez Chialanza et al. (2018), the first step in obtaining different size range of microplastics is achieved by sieving. Sieving helped to obtain a micro plastics sample of homogenized size, the particles were cross checked through the Stereomicroscope (Olympus DSX1000). Figure 10 shows the images taken from microscope of a microplastic fragment having width of 20 microns and length of 13 microns at 280X magnification.

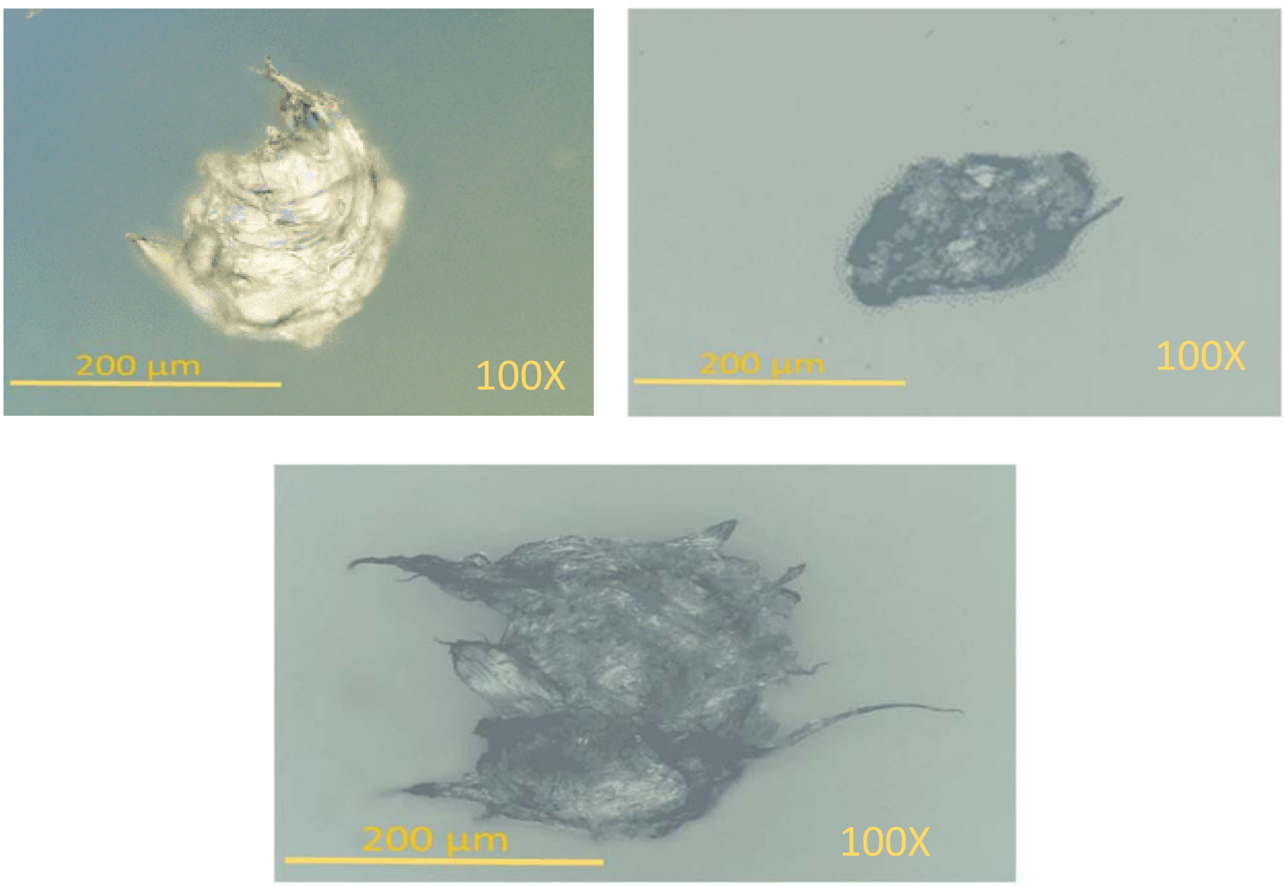


Figure 10: Microplastic fragments, analyzed by stereomicroscope for dimentions.

4.1.2 Particle Size Analysis

Schrank's et al. (2019) highlighted different methods for the analysis of micro and nanoparticles in the environment and one of these methods is laser diffraction technique which measures particles of sizes 500 nm (0.5 microns) to 10 mm (1000 microns). According to Wang et al. (2021), quantification of MPs through a laser diffraction technique is widely used nowadays due to its non-destructive nature.

The results from the particle size analysis revealed that the plastic particles are in the ranges as shown in Table 4, the mean is the size of the particles, which in the below samples ranges from 3.3 to 24 µm, with each samples mode, standard deviation, median and variance

Table 4: Results of particle size analysis

Sr No	Laser Transmittance %	Mean (µm)	Variance (µm)
PP	99.3	31.84	1.87
HDPE	99.5	16.97	100.10
LDPE	99.7	21.76	98.90

Figure 11 shows the particle size distribution, in the figure the **q%** denotes the log of particle diameters percentage and **Q%** denotes the laser percentage that is diffracted by the diameter of the plastic fragments, the q & Q% provides a measure that the sample analyzed has particles between this range, and in microplastics case, a range of particle sizes for further synthetic experiments, in the Figure 1, it is evident that the PP which is analyzed contains microplastic fragments in the range of 1.3-6.7 micrometers with maximum particles of 2.9 micrometers and mean value of 3.38 micrometers. In sample LDPE and HDPE (Figure 3), it is visible that the range of microplastic fragments lies between 2.9-77 micrometers range with maximum particles of 15-34 micrometers with mean values of 24.17 and 24.76 micrometers respectively.

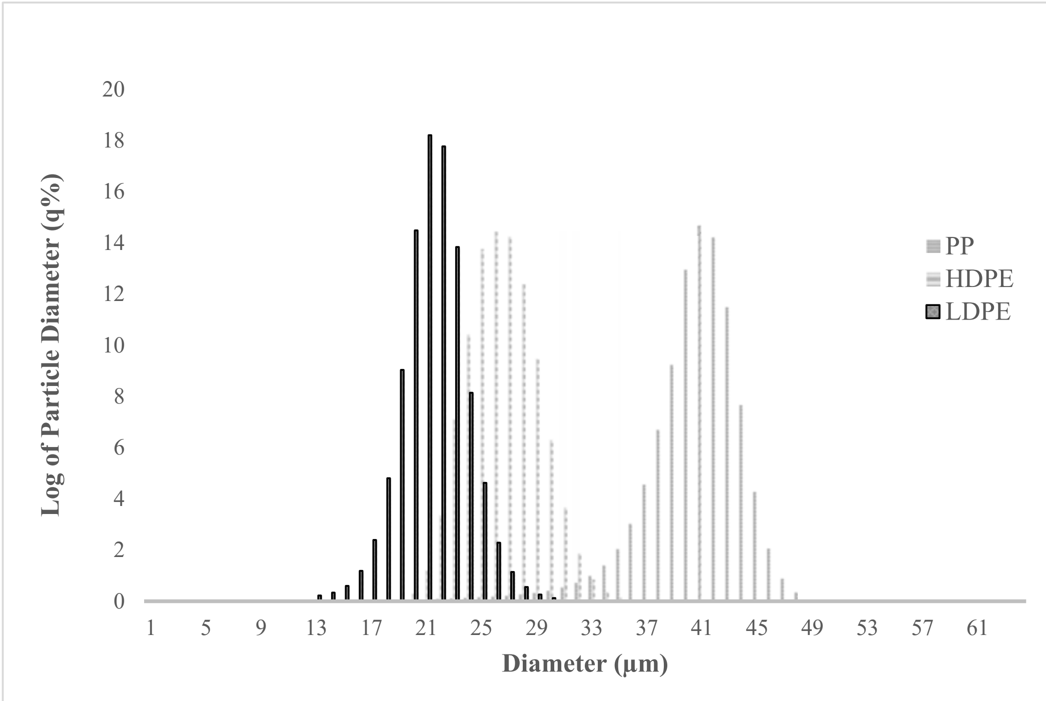


Figure 11: Graph of peaks of LDPE, HDPE and PP after particle size analysis

4.1.3 FTIR Spectroscopy

In recent years, FTIR imaging has demonstrated to be a reliable method for the identification and quantification of microplastics. According to Chen et al. (2020), FTIR technique is now considered as most popular method for MPs identification and quantification and is an ideal tool for automated MPs analysis. Renzi et al. (2019) used FT-IR for the analysis of microplastics in environmental samples due to its reliability. All samples were analyzed in the ATR mode, the wavenumber range of FTIR was 4000-650 cm^{-1}. The *MicroLab* software was used to analyze the results i.e. absorption peaks of samples, software matched the absorbance of the sample with most similar polymer, the standards used for comparing the plastics were industrial standards predefined in the Agilent Handheld ATR library.

After analysis, sample 01 resembled with the Poly propylene standard (Figure 13), sample 02 with Low-density polyethylene (LDPE) (Figure 14) and sample 03 resembled with the High-density polyethylene (HDPE) (Figure 15)

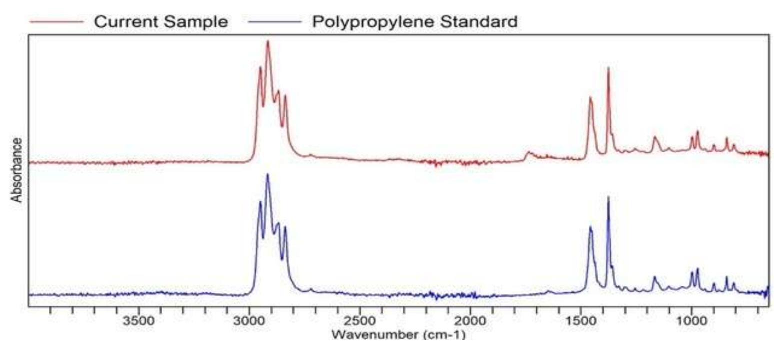


Figure 13:FT-IR spectrum of PP as compared with standard

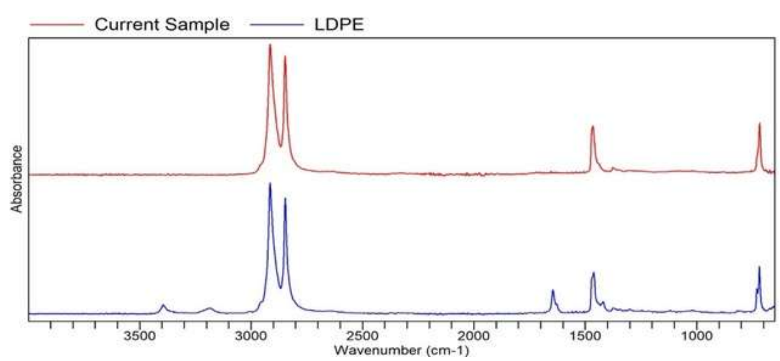


Figure 14: FT-IR spectrum of LDPE as compared with standard

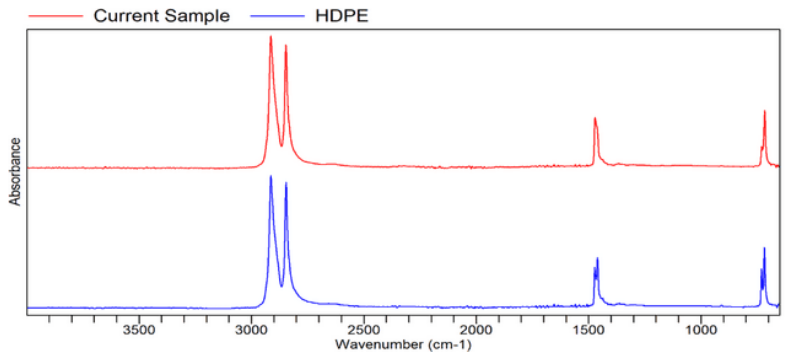


Figure 15: FT-IR spectrum of HDPE as compared with standard

Table 5: Probabilty analysis result by FT-IR

Sample No.	**Resemblance**	**Quality (%)**
1	Poly propylene	96%
2	Low-density polyethylene(LDPE)	97%
3	High-density polyethylene (HDPE)	98%

4.2 Effects of Microplastics on *Daphnia magna*

4.2.1 *Daphnia magna* survival

The LC50 values for three different types of microplastics, namely PP, LDPE, and HDPE, were determined through combined and mono exposure study using the freshwater crustacean *Daphnia magna*. The LC50 value is the concentration of the test substance at which 50% of the exposed organisms die within a given exposure period. The LC50 exposure experiment was run for 48 hours and the results were recorded.

LC50 value for PP was found to be 120, indicating that at a concentration of 120 mg/L of PP microplastics, 50% of the exposed *Daphnia magna* died within the 48 hours exposure period. For LDPE, LC50 value was 107, indicating that the toxicity of LDPE microplastics was higher than that of PP. Finally, LC50 value for HDPE was 123, indicating that toxicity of HDPE microplastics was similar to that of PP. (Figure 16)

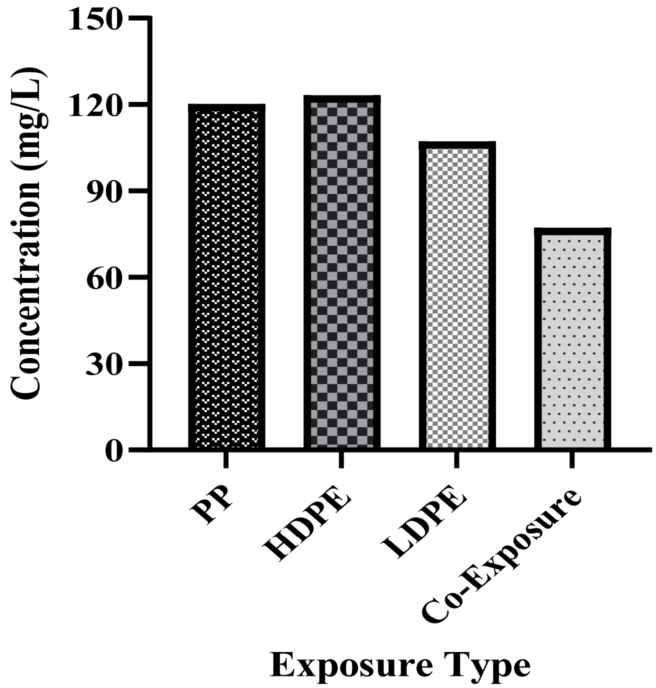


Figure 16: Survival as calculated by LC50

Figure 16 shows the mortality to dose response behaviour of *Daphnia magna*, it was observed that after 48 hours of exposure to the microplastics, significant number of dead *Daphnia magna* were found in co-exposure mixture, as the concentration increased from 30 to 150 mg/L the death rate of *Daphnia magna* magna increased. Similar trend was observed for mono exposure of microplastics, death rate increased with the increase of concentration but there wasn't any significant increase in death rate when compared to the co exposure mixture. The trend of mortality in increasing order was PP<LDPE<HDPE<co-exposure.

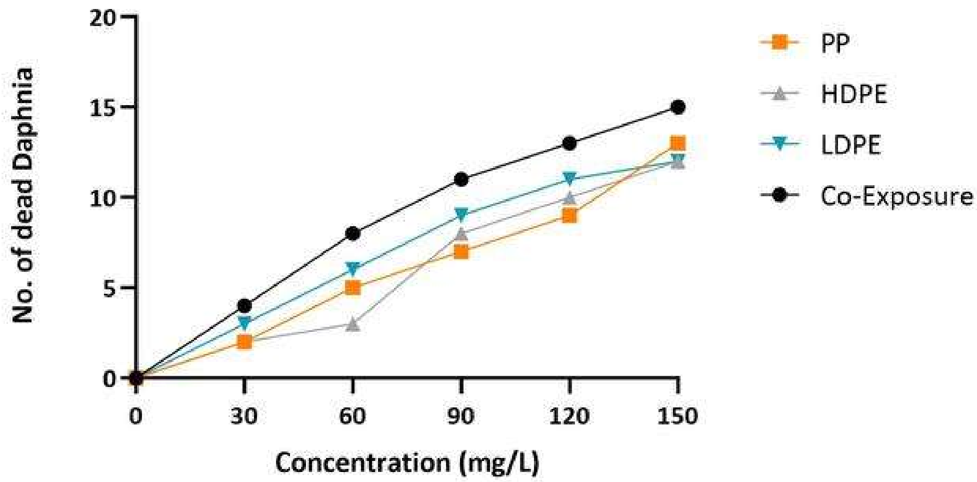


Figure 17: Comparison of dead *Daphnia magna* in various exposure

The LC50 results showed that mono exposure of microplastics of all three types resulted in death of 50% population at concentrations of 120, 123, 107 for PP, HDPE, LDPE, whereas the LC50 for the co-exposure was recorded as 77 mg/L.

In terms of % mortality to *Daphnia magna* due to the mono and co exposure, results are expressed in figure 17, showing that LDPE being more lethal to *Daphnia magna* at lower concentrations 0-90 µg/l than PP and HDPE, however PP is also lethal as compared to HDPE. HDPE showed highest mortality to *Daphnia magna* at higher concentrations (90-150 mg/l) due to the characteristics of HDPE polymer.

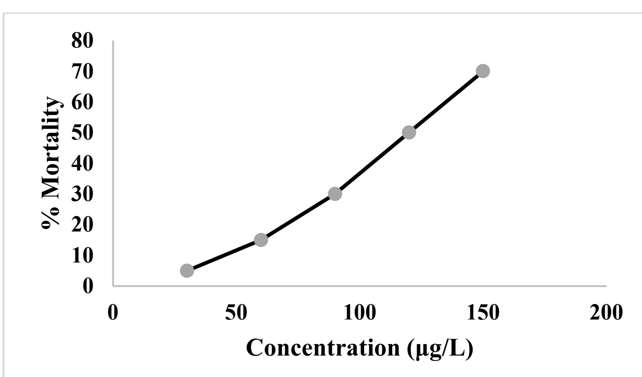


Figure 18: 48 hours LC50 of PP mono exposure

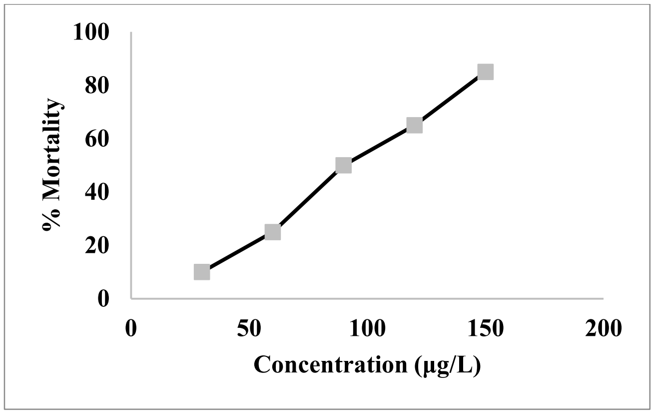


Figure 19:48 hours LC50 of LDPE mono exposure

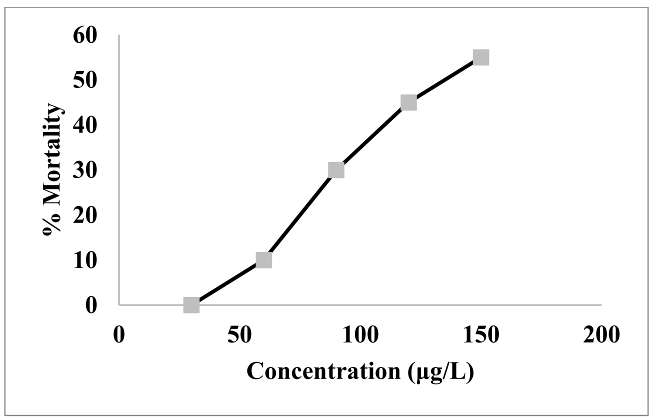


Figure 20: 48 hours LC50 of HDPE mono exposure

exposure of this shows that in realistic scenarios where there are multiple microplastics present in the lake/river or ocean, the plastics are contagious on a lower concentration. Canniff in his study concluded that microplastics of single type have less significant effect on the survival of *Daphnia magna*, they concluded that exposure containing higher quantity of microplastics i.e., 100 mg/L had more effect on the survival of microplastics (Canniff & Hoang, 2018).

Gerdes (2019) observed similar outcomes to those presented in the current study. Employing PET as the primary microplastic polymer, their investigation revealed a diminished survival rate of *Daphnia magna* at an LC50 concentration of 160mg/L (Gerdes et al., 2019). In contrast, the present study demonstrates LC50 values ranging from 107-123 mg/L for various singularly exposed microplastics, while the LC50 value for the synergistic effect of microplastic mixtures was 77mg/L, indicating heightened lethality at lower concentrations. Gardes' experiment concluded that prolonged exposure duration correlates with increased mortality rates. The pervasiveness of the microplastics predicament in real-world environments poses a substantial threat to *Daphnia magna*, as even lower concentrations prove deleterious. This poses a potential hazard to the food chain, given *Daphnia magna*'s pivotal role as a primary food source for predators. Bosker et al. (2019) underscore the importance of recognizing that LC50 values represent just one facet of toxicity assessment, and that other endpoints such as growth, reproduction, and behavior may also be adversely influenced by microplastics.

In current study, microplastics used were irregularly sized fragments, these mimic the marine and freshwater environment more realistically as fragments are the most dominant type of microplastics present in the environment formed by abrasion of the plastics items in the sea. Joorim Na (2021) investigated the effects of microplastic fragments and stated that the fragments alone were 80 time more lethal in an acute experiment than regularly sized microplastics (Na et al., 2021).

4.2.2 Microplastic Ingestion by *Daphnia magna*

The results indicated that the average number of microplastic particles in *Daphnia magna* exposed to HDPE was 8.1, for PP it was 7, and for LDPE it was 6.3. This suggests that HDPE microplastics had the highest concentration in *Daphnia magna*, followed by PP and LDPE. These results register the fact that HDPE which may settle down in the marine environment is more palatable to *Daphnia magna* followed by PP and LDPE, HDPE having high density causes more accumulation in *Daphnia magna* due to vertical swimming patterns of *Daphnia magna*.

Furthermore, results showed that in the co-exposure experiment, the number of microplastic particles in *Daphnia magna* increased to 13, indicating that exposure to multiple types of microplastics could result in a higher concentration of microplastics in aquatic organisms (Figure 21)

The confirmation of microplastic ingestion in both exposure scenarios was established through the digestion procedure. Regarding the singular exposure of microplastics such as LDPE, HDPE, and PP, the highest incidence of ingestion was observed in HDPE, with an average of 8.1 particles per organism, followed by PP and LDPE with 7 and 6.3 particles per *Daphnia magna*, respectively. Canniff's study demonstrated that with increasing concentration, the number of ingested microplastic particles also increased, albeit within a smaller range of 0.8-2 microplastics per *Daphnia magna*, owing to larger particle size (63-75 micrometers) and lower concentration exposures (Canniff & Hoang, 2018).

Conversely, in the context of co-exposure to microplastics, the average number of microplastic particles per *Daphnia magna* was 13, which is notably higher compared to singular exposures. Fabricant (2021) did investigation into the ingestion of polyethylene and polystyrene microplastics underscored the need for models encompassing all microplastic varieties present in natural environments, as opposed to solely synthetic lab-based environments. Their findings indicated that *Daphnia magna* readily ingest both types of microplastics across varied concentrations. The ingestion of microplastics serves as evidence that the primary food sources for predators are becoming increasingly contaminated with plastic particles, potentially leading to the transfer of microplastics throughout the food chain, given the fundamental position of these organisms within it (Fabricant et al., 2021).

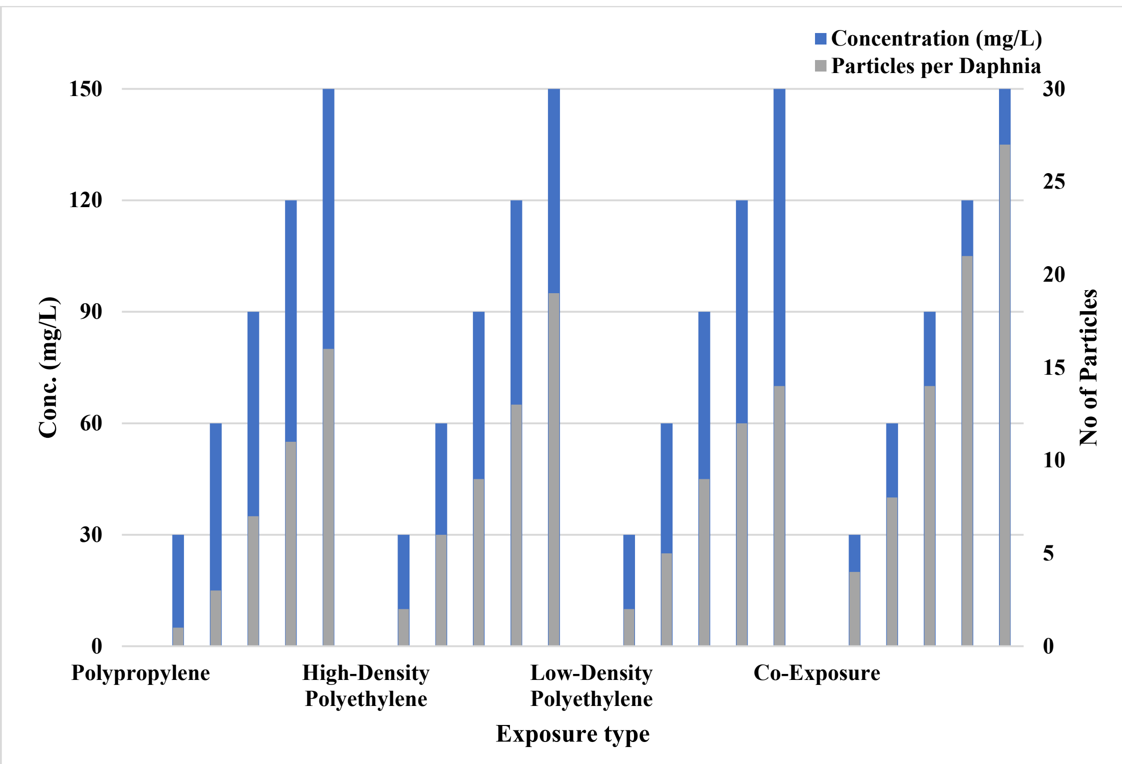


Figure 21: Comparison of Particles per indiviual in both exposure types

Flourescence microscopy technique was used for the quantification of microplastics in the *Daphnia magna* (Figure 22-24) in order to get a clear understanding of how microplastics accumulate inside the gut and other body parts of *Daphnia magna*

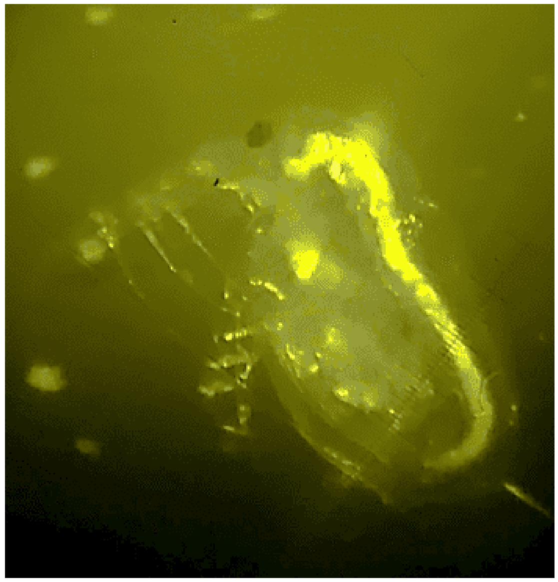


Figure 22: Fluorescently labelled gut of *Daphnia magna* under green light.

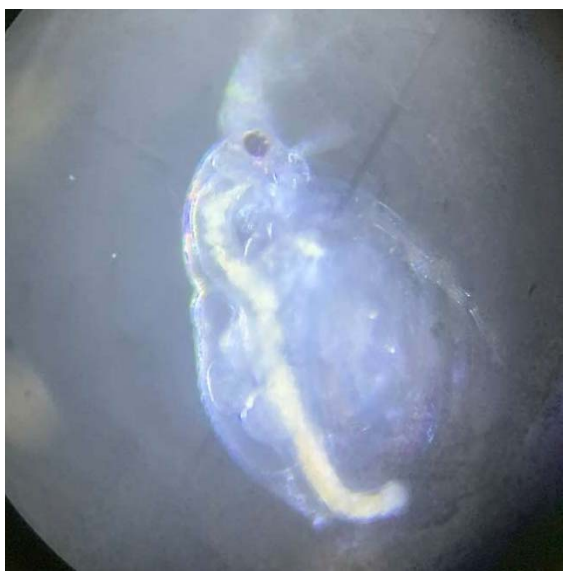


Figure 23: Fluorescently labelled gut of *Daphnia magna* under white light

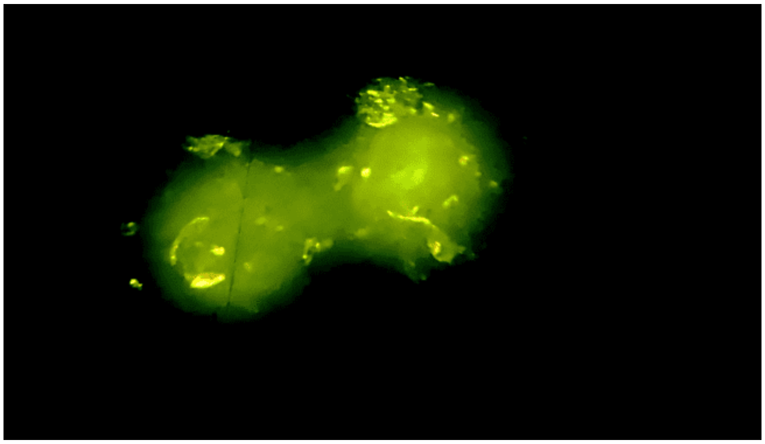


Figure 24: Fluorescently labelled fecal matter of *Daphnia magna* observed under green light

4.2.3 Ingestion in Co-exposure scenario

At a concentration of 30 mg/L, results showed that the ingestion of microplastics was highest for LDPE, followed by HDPE and PP. At a concentration of 60 mg/L, the ingestion of microplastics was also highest for LDPE, followed by HDPE and PP. However, at a concentration of 90 mg/L, the ingestion of microplastics was highest for HDPE, followed by LDPE and PP.

Furthermore, at a concentration of 120 mg/L, the study found that HDPE was the most prevalent type of microplastic ingested by *Daphnia magna*. Finally, at a concentration of 150 mg/L, PP was found to be the most prevalent type of microplastic ingested by *Daphnia magna*.

These results (Table 6) are one of a kind results which are published by this study, there has been limited work done on the co-exposure of microplastics. Although extensive research has been carried out on the topic of the sorption of microplastics with contaminants or feeding a mixture of microplastics with algae etc. but this research provide the insight about how *Daphnia magna* consumes different microplastic polymers and whats the maximum uptake at a given concentration.

Table 6: Abundance of different microplastic types in *Daphnia magna* after digestion and analysis by FT-IR.

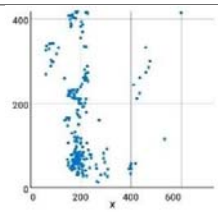
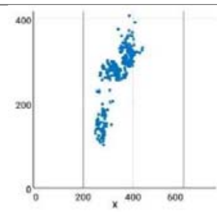
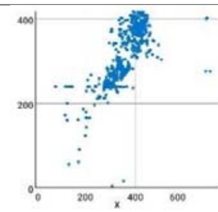
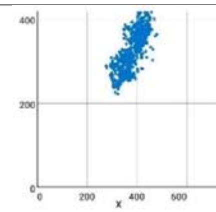
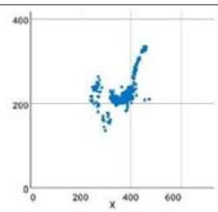
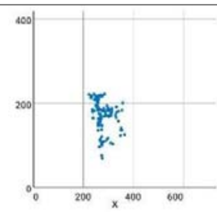
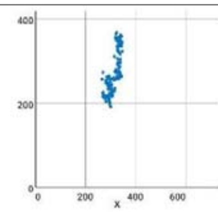
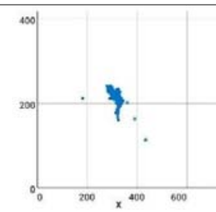
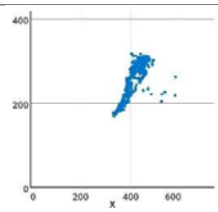
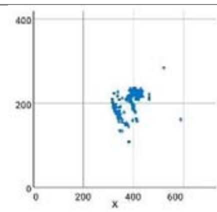
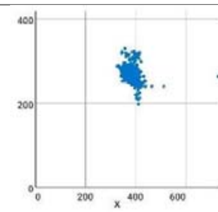
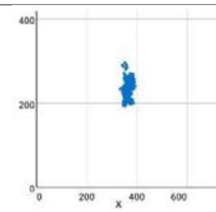
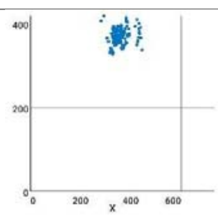
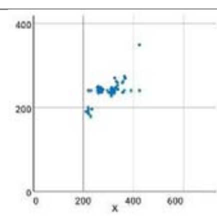
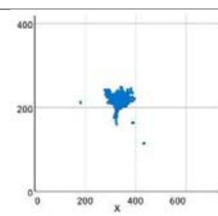
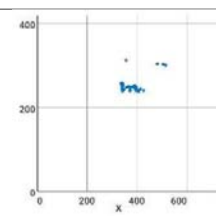
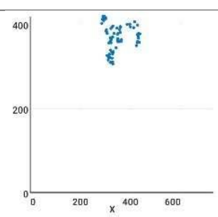
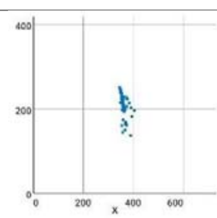
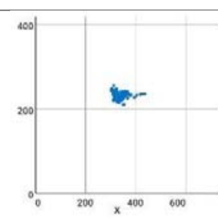
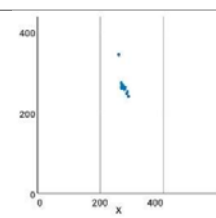
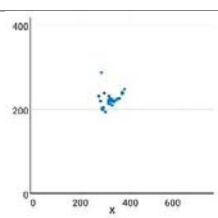
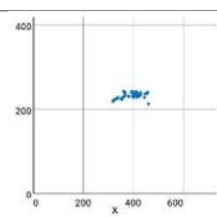
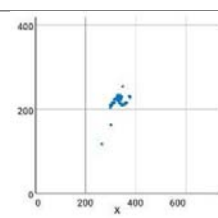
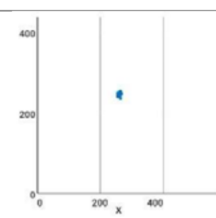
Conc. (mg/L)	Polymers present	Abundance
0	None	None
30	LDPE,HDPE,PP	**LDPE > PP > HDPE**
60	LDPE,HDPE,PP	**LDPE > PP > HDPE**
90	LDPE,HDPE,PP	**LDPE > HDPE > PP**
120	LDPE,HDPE,PP	**HDPE > PP > LDPE**
150	LDPE,HDPE,PP	**PP > HDPE > LDPE**

4.2.4 *Daphnia magna* swimming behavior

This study aimed to investigate the swimming behavior of *Daphnia magna* under exposure to three distinct microplastic types: HDPE, PP, and LDPE, both individually and in combination. The findings revealed a notable deceleration in the swimming activity of *Daphnia magna* upon exposure to HDPE, PP, and LDPE microplastics individually, in comparison to the control group. Particularly, the swimming velocity of *Daphnia magna* exposed to HDPE experienced the most substantial reduction, while for PP and LDPE, the effect was comparatively milder, though still discernible in contrast to the control group. These outcomes signify a detrimental influence of various microplastic types on the swimming behavior of *Daphnia magna*.

Moreover, in the co-exposure scenario, the swimming behavior of *Daphnia magna* faced further deterioration, manifesting an augmented immobility. Instances of complete immobility were observed among *Daphnia magna*, with no discernible active movements, indicating a significant decline in swimming behavior compared to both the control group and the singular exposures.

Table 7: Swimming behavior analysis of all microplastic concentrations by AnimApp

Exposure Conc. (mg/L)	PP	LDPE	HDPE	Synergystic
0				
30				
60				
90				
120				
150				

The swimming behavior of *Daphnia magna* is often reported in many studies due to its relation with being an indicator of stress, the current study analyzed the swimming behavior of *Daphnia magna* with the help of a software and through visible introspection. The swimming activity of *Daphnia magna* is one of its characteristic traits which help other organisms in the food chain obtain food and its predator prey interaction depends on the swimming of the organism. The mono exposures of *Daphnia magna* showed the reduction in speed, increased immobility and reduction in filtering ability, the maximum effects were observed for LDPE followed by PP and HDPE. The spinning of organisms in a similar pan was observed in higher concentrations while in blank there was crusing, hoping and sinking movements which indicates that the presence of single type of microplastics increased the stress on the organism. investigated the effects of PP microplastics on the swimming behavior of *Daphnia magna*. The study by Magester (2021) found that exposure to PP microplastics (1-1000 µm) resulted in a significant decrease in swimming velocity and increased erratic movements in *Daphnia magna* (Magester et al., 2021). investigated the effects of HDPE microplastics on the swimming behavior of *Daphnia magna*. Another study found that exposure to HDPE microplastics (10-1000 µm) resulted in a significant decrease in swimming velocity and increased turning behavior in *Daphnia magna* (Na et al., 2021) choi et al investigated the effects of LDPE microplastics on the swimming behavior of *Daphnia magna*. The study found that exposure to LDPE microplastics (1-100 µm) resulted in a significant decrease in swimming velocity and increased turning behavior in *Daphnia magna* (Choi et al., 2018).

In the context of co-exposure, previous studies have not explored the alterations in the swimming behavior of Daphnia magna. This study stands out as pioneering in its examination of the synergistic effects of various microplastic types on Daphnia magna. It was noted that Daphnia magna exhibited spinning and erratic movements up to an exposure concentration of 90 mg/L; however, beyond this concentration, the presence of diverse microplastic types induced immobility in the organism, a phenomenon exclusively observed in co-exposure scenarios and not in any singular exposure instances. Complete immobility serves as a clear indicator of heightened stress levels and severely depleted energy reserves, suggesting that microplastics have interfered with the organism's appendages and chemosensory organs. Consequently, the capacity for movement and food detection is significantly compromised in the case of co-exposure (Canniff & Hoang, 2018b; Galloway et al., 2017; Junaid et al., 2023).

4.2.5 Population density

Our findings revealed that exposure to each type of microplastic individually led to a decrease in the population density of Daphnia magna. Specifically, the average population density of Daphnia magna exposed to HDPE, PP, and LDPE was 2 individuals per 10 mL, 2.1 individuals per 10 mL, and 2.2 individuals per 10 mL, respectively, in contrast to the control group's average population density of 3.5 individuals per 10 mL. These results underscore the adverse impact of different microplastic types on the population density of Daphnia magna. Furthermore, in the co-exposure scenario, the population density of Daphnia magna experienced a further reduction. The average population density of Daphnia magna exposed to multiple microplastic types was 0.6 individuals per 10 mL, indicating a significant decrease compared to both the control group and individual exposure scenarios.

The decline in Daphnia magna population density over the course of five days can be attributed to frequent organism deaths, compounded by factors such as smaller population sizes, which yielded quicker observable results. However, notably, the population density decreased more sharply in the case of co-exposure compared to singular exposures, confirming our findings regarding the heightened danger posed by the synergistic effects of microplastics on Daphnia magna. Similar conclusions were drawn by Schrank et al. (2019) in their study. In another study by Jeyavani (2020), it was reported that the population density decreased in the presence of polypropylene microplastics, as the microplastics adhered to the bodies of Daphnia magna, hindering egg laying and subsequently leading to a decline in population density. Similarly, Schöpfer et al. (2020) observed a reduction in the population density of nematodes due to microplastic ingestion, accompanied by a decrease in egg laying rates.

4.2.6 *Daphnia magna* reproduction rate

Study results showed that exposure to each type of microplastic individually resulted in a reduction in the reproduction rate of *Daphnia magna*. Specifically, average reproduction rate of *Daphnia magna* exposed to HDPE was 1.2 offspring per individual, for PP it was 1.6 offspring per individual, and for LDPE it was 1.5 offspring per individual, compared to the control group which had an average reproduction rate of 2.1 offspring per individual. These results indicate that exposure to different types of microplastics may have a negative impact on the reproduction rate of *Daphnia magna*.

Furthermore, in the co-exposure scenario, reproduction rate of *Daphnia magna* was further reduced. The average reproduction rate of *Daphnia magna* exposed to multiple types of microplastics was 0.8 offspring per individual, indicating a significant decrease in reproduction rate compared to the control group and individual exposure scenarios.

Table 8: Reproduction rate of *Daphnia magna* at different concentrations

Exposure Conc. (MG/L)	PP	LDPE	HDPE	Co-Exposure
30	2	1.85	1.57	1.5
60	2	1.83	1.5	1.3
90	1.7	1.6	1.2	1.3
120	1.5	1.5	1	0
150	1	1	1	0
Average	**1.64**	**1.556**	**1.254**	**0.82**

Daphnia magna are renowned for their prolific reproductive capabilities, often doubling or even tripling their population within a span of 3-4 days under optimal environmental conditions. In the present study, both mono and co-exposure experiments focused on assessing the reproduction rate of these organisms, serving as an indicator of their behavior under stress conditions. Reproduction rate calculations were based on neonates and adult *Daphnia magna* present in the exposure solution, employing a methodology akin to that of Canniff et al. (2018). The normal reproduction rate was determined to be 2.2, with the reproduction rates of PP, LDPE, and HDPE measured at 1.6, 1.5, and 1.3, respectively, in mono exposure scenarios. Notably, HDPE exhibited the lowest reproduction rate, followed by LDPE and PP. This trend aligns with the findings of Canniff and colleagues (2018), who reported an average reproduction rate of 1.2 for polyethylene, consistent with the results of the current study, which utilized microplastic fragments of smaller size (32 micrometers).

In the context of co-exposure to microplastics, the reproduction rate plummeted to 0.8, a stark decrease of three times compared to the normal rate. Microscopic observations revealed that although the brood chambers of *Daphnia magna* contained 2/3 neonates, the organisms were unable to deliver them due to adverse external conditions unsuitable for neonate laying. Factors such as a gut filled with microplastics and continuous starvation induced by the synergistic effects of microplastics contributed to this observed phenomenon. Additionally, the ability of movement was hampered, and the energy reserves of *Daphnia magna* depleted more rapidly compared to other mono exposures.

Several prior studies have delved into the effects of microplastics on reproduction in *Daphnia magna*. For instance, Jemec et al. (2016) found that exposure to microplastics led to reduced fecundity in *Daphnia magna*, resulting in fewer offspring per brood. Similarly, Yuan et al. (2018) reported alterations in the sex ratio of offspring produced by *Daphnia magna* following exposure to microplastics, with a higher proportion of males being produced.

Figure 26: Eggs inside *Daphnia magna* for reproduction purpose observed under light microscope

4.2.7 Body length of *Daphnia magna*

Results showed that exposure to each type of microplastic individually resulted in a reduction in the body length of *Daphnia magna*. Specifically, average body length of *Daphnia magna* exposed to HDPE was 710 µm, for PP it was 730 µm whereas for LDPE it was 670 µm, compared to the control group which had an average body length of 960 µm. These results indicate that exposure to different types of microplastics may have a negative impact on the body length of *Daphnia magna*. Furthermore, in the co-exposure scenario, body length of *Daphnia magna* was further reduced. The average body length of *Daphnia magna* exposed to multiple types of microplastics was 550 µm, indicating a significant decrease in body length compared to the control group and individual exposure scenarios.

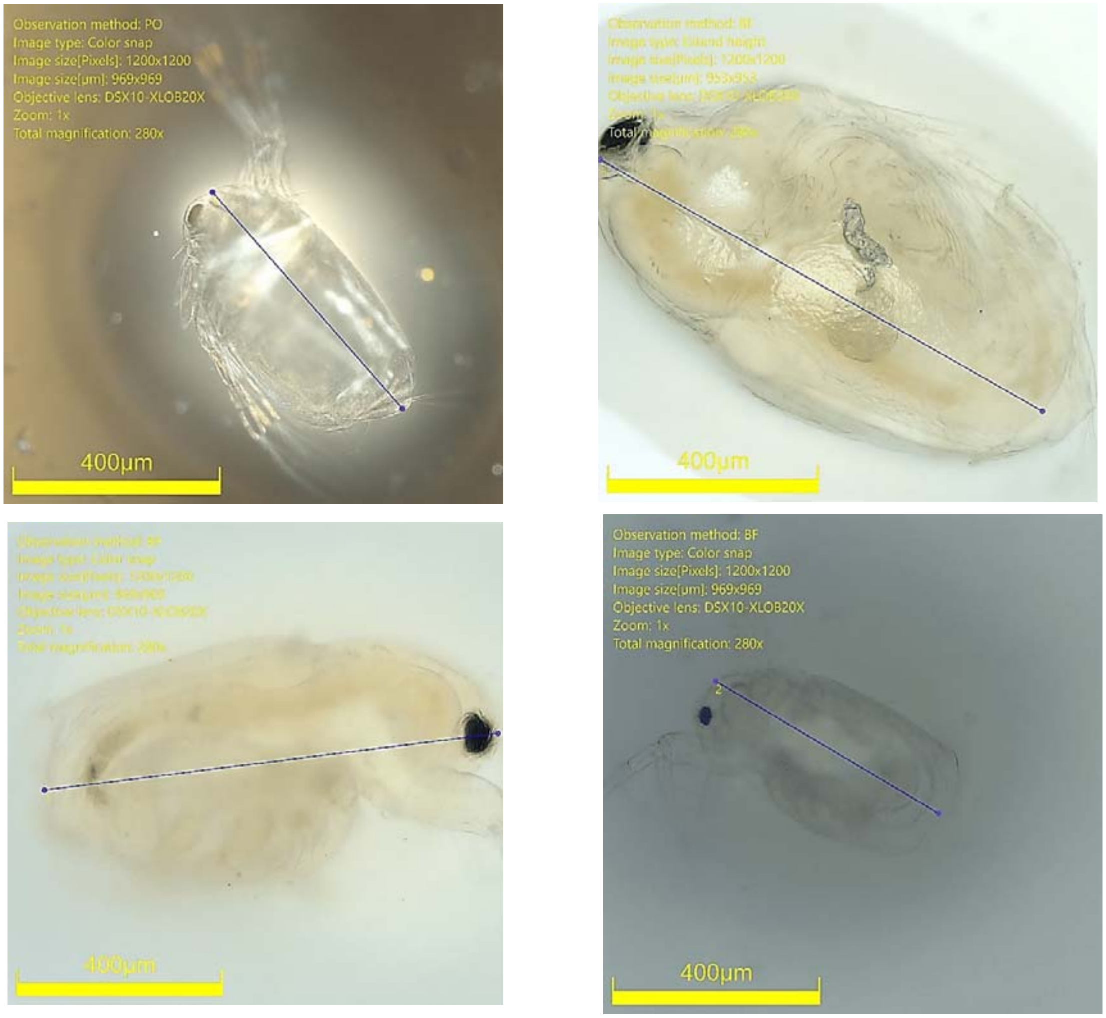


Figure 27: Body length measurements of *Daphnia magna* magna under stereomicroscope.

A reduction in body length was observed across various exposure scenarios, with PP exposure resulting in a 24% decrease, LDPE exposure leading to a 31% reduction, HDPE exposure causing a 26% decrease, and a more pronounced 43% reduction observed in the case of co-exposure. This trend aligns with findings by Castro et al. (2020), who reported a decrease in *Daphnia magna* body length ranging from 40-43% due to exposure to microplastics sized 1-48 microns, albeit at higher concentrations (320 mg/L compared to 150 mg/L in this study). Furthermore, Renzi et al. (2019) observed similar outcomes in their study, where the presence of microplastics in exposure solutions led to a reduction in the body length of *Daphnia magna*.

Additionally, research conducted by Imhof et al. (2017) highlighted a decrease in the length of the digestive tract of *Daphnia magna* in response to the presence of microplastics, corroborating the findings of the current study. These collective findings underscore the detrimental impact of microplastics on the morphology and physiology of *Daphnia magna*, including alterations in body length and digestive tract length.

Table 9: Exposure and body length relationship at different concentrations

Type of exposure	Body length (µm)
PP	730
LDPE	670
HDPE	710
Co-exposure	550
Normal Daphnia	>960

4.2.8 Egestion rate

Results showed that *Daphnia magna* exposed to microplastics had a reduced egestion rate compared to control group. In control group, 8-10 fecal pellets were produced per organism, while in mono-exposure scenario, 6-8 fecal pellets were produced. This indicates that exposure to microplastics may reduce the egestion rate of *Daphnia magna*.

Interestingly, in the co-exposure scenario, the reduction in egestion was more pronounced, with only 3-4 fecal pellets produced per organism. This suggests that the combined exposure to different types of microplastics may have a synergistic effect on the egestion behavior of *Daphnia magna*.

The reduced egestion rate observed in this study has several potential implications for the ecological health of aquatic ecosystems. Egestion is an important process in the transfer of nutrients and energy through the food chain, and a decrease in egestion rate may result in a decrease in nutrient recycling and productivity. Egested fecal pallets are food for other organisms in the marine ecosystem, if microplastics are present in these fecal pallets, there is a high chance that plastics will accumulate in other organisms and will transfer in environment as reported by Cole (2016) in their research, in which they provided evidences of microplastics found in egested pallets, these microplastics will now circulate in the environment through various biological processes and will take years to degrade (Cole et al., 2016).

In a study investigating the fecal settling density of zooplankton, conducted by Shore and colleagues, it was found that the presence of microplastics within fecal pellets led to a decrease in settling time. This phenomenon resulted in fecal pellets being submerged in the water column, where they were readily consumed as food by other organisms. Such findings suggest a potential disruption to the food web, as the presence of microplastics in fecal matter can inadvertently introduce these pollutants into the diet of other organisms, thus posing a threat to the integrity and balance of the ecosystem (Shore et al., 2021).

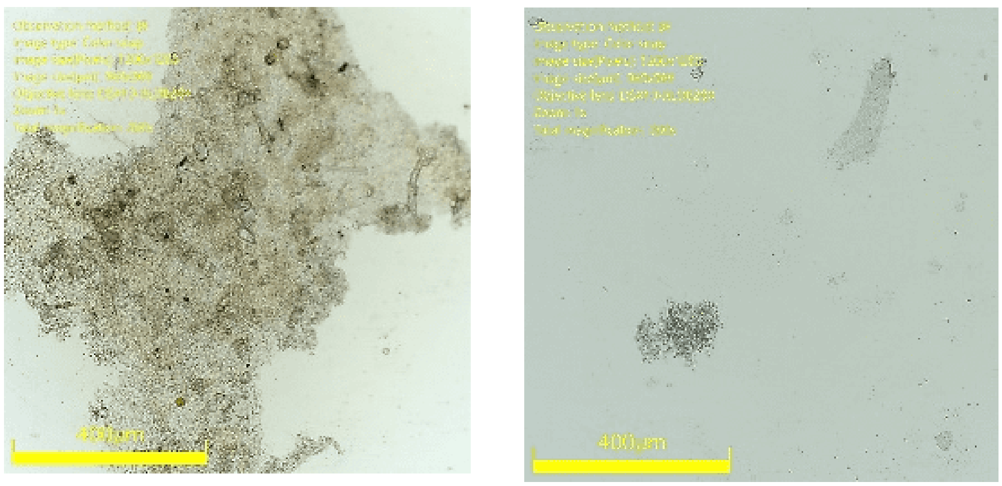


Figure 28: Feces of *Daphnia magna* in different exposure scenarios

CHAPTER 5

CONCLUSION & RECOMMEDNATIONS

In **conclusion**, this study sheds light on the profound effects of microplastic exposure on *Daphnia magna*, a critical organism in aquatic ecosystems. Laboratory-synthesized microplastics of three plastic polymers (PP, HDPE, LDPE) demonstrated high purity within a size range of 0-32 microns, emphasizing the relevance of these findings to real-world scenarios. Mono exposure experiments unveiled a spectrum of adverse impacts, including reduced survival thresholds, altered behaviours, diminished reproductive success, and physical changes in the organisms. Co-exposure scenarios exacerbated these effects, demonstrating a 20% increase in toxicity and leading to significant declines in reproduction rate, alongside heightened behavioral changes and increased accumulation of microplastics within organisms. Given these findings, it is imperative to conduct extended studies focusing on cumulative and chronic impacts of microplastic exposure on *Daphnia magna*, while considering various plastic polymers and assessing broader ecological consequences. Understanding the synergistic effects of co-exposure scenarios and designing studies that reflect realistic environmental conditions are crucial steps toward comprehensively addressing the microplastic pollution crisis. Furthermore, investigating the influence of microplastic size on toxicity, bioavailability, and ingestion rates will provide valuable insights into mitigating the impact of microplastics on aquatic ecosystems and the broader environment.

Recommendations include to conduct extended studies on microplastic exposure effects on Daphnia magna, focusing on cumulative and chronic impacts and include various plastic polymers and assess broader ecological consequences. Investigate synergistic effects in co-exposure scenarios to understand how different plastic polymers interact and amplify toxicity and design studies reflecting realistic environmental conditions. Explore the influence of microplastic size on toxicity, bioavailability, and ingestion rates, considering the range of particle sizes in aquatic ecosystems along with food chain contamination caused by microplastics

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