ACUTE EXPOSURE OF PP, LDPE & HDPE MICROPLASTICS ON MARINE MICRO-CRUSTACEAN (*Artemia salina*)

By

Mariam Ali

(Registration No: 00000328867)

Institute of Environmental Sciences and Engineering

School of Civil and Environmental Engineering

National University of Sciences & Technology (NUST)

Islamabad, Pakistan

(2024)

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A thesis submitted to the National University of Sciences and Technology, Islamabad, in partial fulfillment of the requirements for the degree of

Master of Science in Environmental Sciences

Supervisor: Dr. Hira Amjad

Co-Supervisor: Dr. Imran Hashmi

Institute of Environmental Sciences and Engineering

School of Civil and Environmental Engineering

National University of Sciences & Technology (NUST)

Islamabad, Pakistan

(2024)

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Signature: Hira Amjad

Name of Supervisor: Dr. Hira Amjad

Date: 06-06-2024

Dr. Zeshan
Tenured Assoc Prof
HoD Environmental Sciences
IESE (SCEE) NUST Islamabad

Signature (HOD): _____

Date: 06/06/2024

Prof. Dr. Imran Hashmi
Associate Dean
IESE (SCEE) NUST Islamabad

Signature (Associate Dean): _____

Date: 06-06-2024

Signature (Principal & Dean SCEE): _____

PROF DR MUHAMMAD IRFAN
Principal & Dean
SCEE, NUST

Date: 07 JUN 2024

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Examination Committee Members

1. **Dr. Muhammad Ansar Farooq** Signature: _____
2. **Dr. Rashid Iftikhar** Signature: _____

Supervisor: **Dr. Hira Amjad** Signature: _____

Co- Supervisor: **Dr. Imran Hashmi** Signature: _____

Dr. Zeshan
Tenured Assoc Prof
HoD Environmental Sciences
IESE (SCEE), NUST Islamabad

Signature HOD: _____ Date: 06/06/2024

COUNTERSIGNED

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Student Name: **Mariam Ali** Signature: _____

Guidance & Examination Committee:

a) **Dr. Muhammad Ansar Farooq** Signature: _____

Associate Professor SCEE (IESE)

b) **Dr. Rashid Iftikhar** Signature: _____

Assistant Professor SCEE (IESE)

Supervisor: **Dr. Hira Amjad** Signature: _____

HOD: **Dr. Zeshan Sheikh** Signature: _____

Associate Dean: **Dr. Imran Hashmi** Signature: _____

Principal & Dean SCEE: Signature: _____

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DEDICATION

“O My Sustainer, bestow on my parents your mercy even as they cherished me in my childhood”.

This research is dedicated to my affectionate and caring parents, siblings and my esteemed teachers, whose diligent efforts and selflessness have transformed my aspiration of obtaining this degree into a tangible achievement. I am profoundly grateful to them, and words are insufficient to convey my appreciation.

ACKNOWLEDGEMENTS

All praises are due to Allah SWT, the one deserving of all recognition and gratitude. His blessings and favor have enabled me to reach this significant milestone.

I express my deepest appreciation, first and foremost, to Dr. Hira Amjad, whose expert guidance was instrumental throughout my research journey. Her unwavering passion for research consistently kept me engaged in my work, and her personal generosity enriched my experience at SCEE (IESE), NUST, contributing to my personal growth.

I extend sincere appreciation to my co-supervisor, Prof. Dr. Imran Hashmi (IESE), for graciously allocating his valuable time to engage in progress presentations and offering substantive recommendations to augment both the research endeavor and the refinement of the thesis composition.

I also acknowledge the vital support and constructive feedback provided by my Guidance and Examination Committee (GEC), comprised of Dr. Ansar Farooq and Dr. Rashid Iftikhar.

Above all, I am profoundly grateful to my family, my parents and brothers, whose significance in my life continues to grow with time. They have been my unwavering source of motivation, and none of this achievement would have been possible without their support.

My appreciation further extends to my colleagues in the laboratory, who contributed to maintaining a positive work environment. Lastly, I would also like to extend my thanks to all my dear friends – Ebadat Ur Rehman, Hajra Maalik, Momina Mailk, Ahmad Hassan, Ghazi Akbar, Haleema Ayaz, Arwa Raza, Hassan Shafiq and Saba Imran whose companionship and support have made my time at NUST truly memorable.

With immense gratitude, I conclude this acknowledgement.

Mariam Ali

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Abstract:

The contamination of marine ecosystems due to microplastics pollution represents a critical environmental threat. This study aims to conduct a comprehensive assessment and comparative analysis of the potential risks posed by individual and combined microplastics on the filter-feeding micro crustacean, *Artemia salina*. Recognizing the extensive presence of microplastics in the environment, this research specifically focused on evaluating the impacts of three prominent microplastics types, Low-Density Polyethylene (LDPE), Polypropylene (PP), and High-Density Polyethylene (HDPE) commonly encountered in aquatic environments. The research findings showed significant variations in the lethal concentration (LC50) values among individual microplastics. Notably, PP, HDPE, and LDPE exhibited LC50 values of 124, 107, and 103 mg/l, respectively. Remarkably, when all three microplastics coexisted, there was a substantial reduction in the LC50 value to 68.2 mg/l. This decrease indicated an elevated mortality rate among *Artemia salina*, underscoring the compounded stress imposed by the simultaneous presence of these plastic polymers. This suggested that combined exposure to multiple microplastics may pose a more severe threat to the survival of *Artemia salina* than exposure to each type individually. Swimming behavior was significantly affected as microplastics concentrations rise, in both mono and co-exposure scenarios. Bioaccumulation analysis indicates comparable microplastics accumulation at lower concentrations, but higher concentrations lead to increased accumulation in *Artemia salina* due to heightened environmental microplastics abundance.

1. INTRODUCTION

1.1. Plastic pollution

Alarmingly, plastic trash makes up > 90% of all marine debris worldwide, which not only disturbs the aesthetics but also has negative effects on the marine ecosystem. (Derraik, 2002). Microplastic pollution in the waters has received more and more attention recently as a major ecological issue on a global scale (Andrady, 2011). Over 250,000 tonnes of plastic and 5 trillion pieces of plastic are estimated to be sinking in the oceans. Because most plastic polymers are durable and resistant to biodegradation, they may last for decades or even centuries, making the environmental lifespan of plastics unknown. The production of plastic surged in the twenty-first century, rising from 200 million tonnes in 2002 to 311 million tonnes in 2014. This could increase to 33 billion tonnes in 2050 (Rochman et al., 2013).

1.2. Status of plastic pollution in Pakistan

0.6 million tons of plastic is produced from around 6000 producers in Pakistan and contributes about 0.2 million tons of plastic waste into the Arabian Sea through the Mighty Indus (Dawn 2019). Polyethene bags are frequently used to transport various goods from the market. Annually, up to 55 billion plastic bags are utilized for this purpose (The News 2018). The International Trade Administration (ITA) estimates that plastics make up about 6% of Pakistan's total solid waste production (48.5 million tonnes), with the majority of the waste being dumped in public areas (International Trade Administration 2019). Alongside various water systems, such as canals, drains, and rivers, open dumping of municipal solid trash is common.

Despite the country's significant plastic consumption and trash creation, Pakistan falls behind and the precise number of plastics in aquatic systems and MPs pollution from surface water is mainly underestimated. The number of macroplastics in the Ravi River was counted during the survey. Polystyrene fragments were also detected floating close to flow regulating gates of barrages, with an average density of 10–30 pieces/m^3 being seen in various areas of the river. However, it was discovered that the river was observably clean and that microplastics were not a significant issue (Irfan et al., 2020).

1.3. Micro plastics

Plastics are artificial polymers that may be molded into a variety of shapes due to their malleable (flexible) nature. Long chains of polymers made of carbon, oxygen, hydrogen, silicon, and chloride, which are derived from natural gas, oil, and coal, make up plastic (Shah et al., 2008). Polyethylene (PE), polypropylene (PP), polystyrene (PS), polyethylene terephthalate (PET), polyvinyl chloride (PVC), low density polyethylene (LDPE), and high density polyethylene (HDPE) are the most popular synthetic polymers and account for 90% of global plastic production (Andrady & Neal, 2009). Plastics are a commonly used material due to their attributes like flexibility, toughness, affordability, ease of handling (lightweight), and corrosion resistance.

Plastic has a significant industrial and commercial usage because it can handle high levels of electrical and thermal insulation (Thompson et al., 2009). From 1950 (1.5 million tonnes) until 2015, plastic output increased exponentially (322 million tons) (Plastics Europe, 2015). Because plastic materials are durable and corrosion-resistant, proper disposal of them is a problem today. It can take years for plastic compounds to break down into smaller pieces (Barnes et al., 2009). Due to shifting environmental circumstances, larger plastic trash gradually breaks down into smaller bits with sizes ranging from meters to micrometers. Microplastics are these broken pieces of plastic that are smaller than 5 mm in size and are very persistent in the ecosystem (Sighicelli et al., 2018).

1.4. Types of microplastics

Microplastics are divided into two groups based on their sources (Avio et al., 2017), these are as follow:

- Primary microplastics
- Secondary microplastics

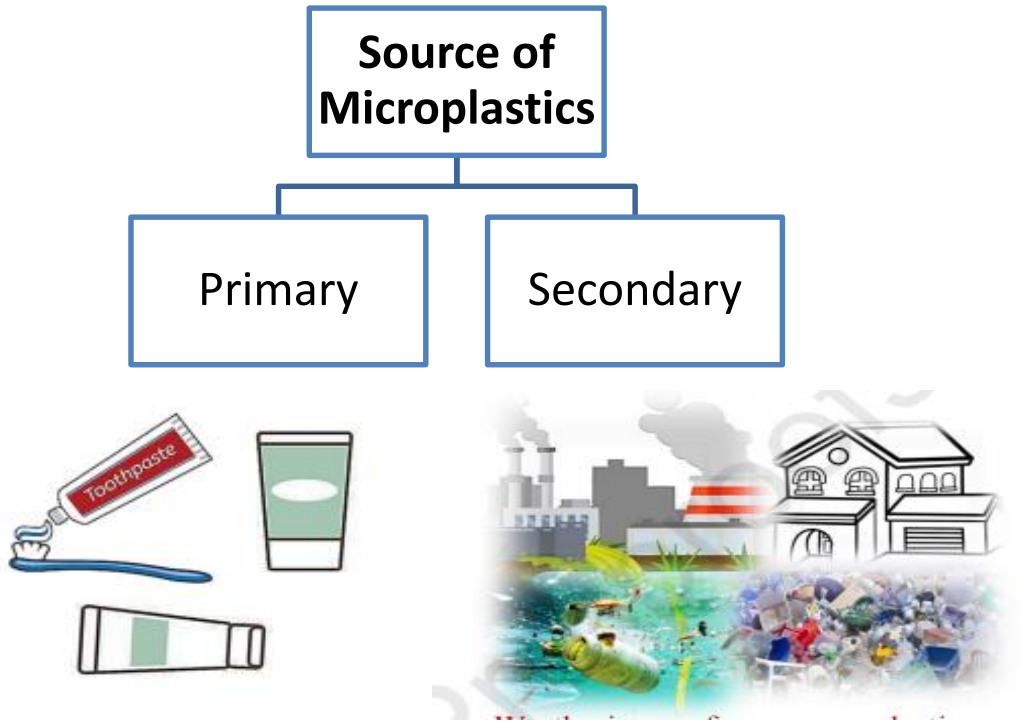


Figure 1.1: Sources of microplastics in the environment

1.1.1. Primary microplastics

Primary microplastics are synthetic polymers that are microscopic in size and are utilized as exfoliates in a variety of processes, including chemical formulation, sandblasting media, plastic product maintenance, and the production of synthetic clothing. Microbeads are a different class of primary plastics (size less than 2 mm) made of polyethylene (PE), polypropylene (PP), and polystyrene (PS) beads and are used in cosmetic and healthcare applications.

1.1.2. Secondary microplastics

Secondary microplastics, which are produced primarily as a result of environmental processes such microbial degradation, photo catalysis, thermal degradation, and hydrolysis, are the fragmented outcome of macro or meso plastics as shown in Figure 1.2 (Sharma & Chatterjee, 2017). Additionally, all microplastics may have ramifications for the bioaccumulation of different chemicals and pollutants (da Costa et al., 2016).

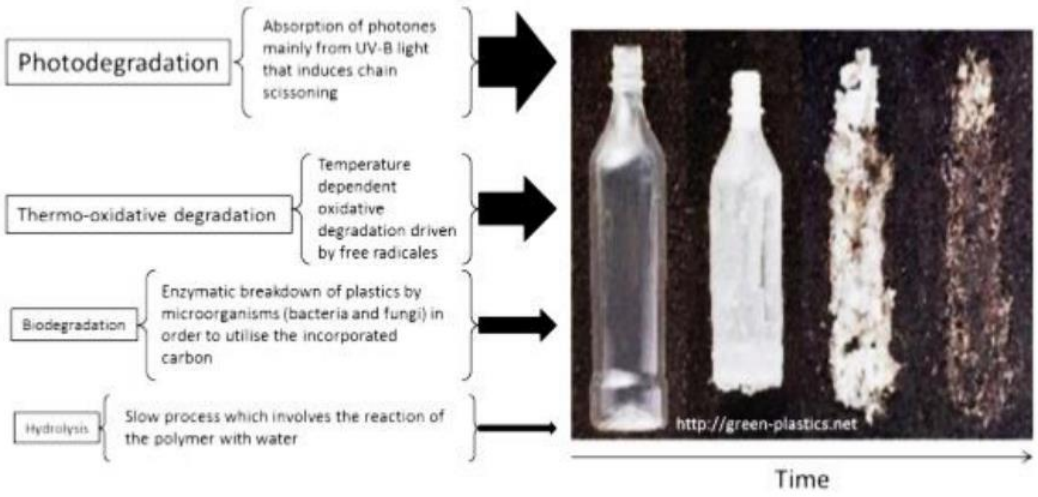


Figure 1.2: Degradation of plastic over time

1.5. MPs in aquatic and marine life

Microplastics endanger aquatic organisms because they can be mistakenly ingested with food (Galloway et al., 2017; Steer et al., 2017). Microplastics may have different effects on animals living in different segments of the marine environment and with different feeding techniques. Furthermore, depending on sensitivity, different life stages of aquatic animals may respond differently to microplastics exposure, with larvae being the most vulnerable (Messinetti et al., 2018). Microplastic particles can enter aquatic organisms' circulatory systems and even accumulate in their guts (Grigorakis et al., 2017). Many harmful effects have been reported in many aquatic animals as a result of plastic accumulation, ranging from physical injury to toxicities of growth and

reproduction (Cole et al., 2015; Sussarellu et al., 2016), including invertebrates, fish, and shrimps.

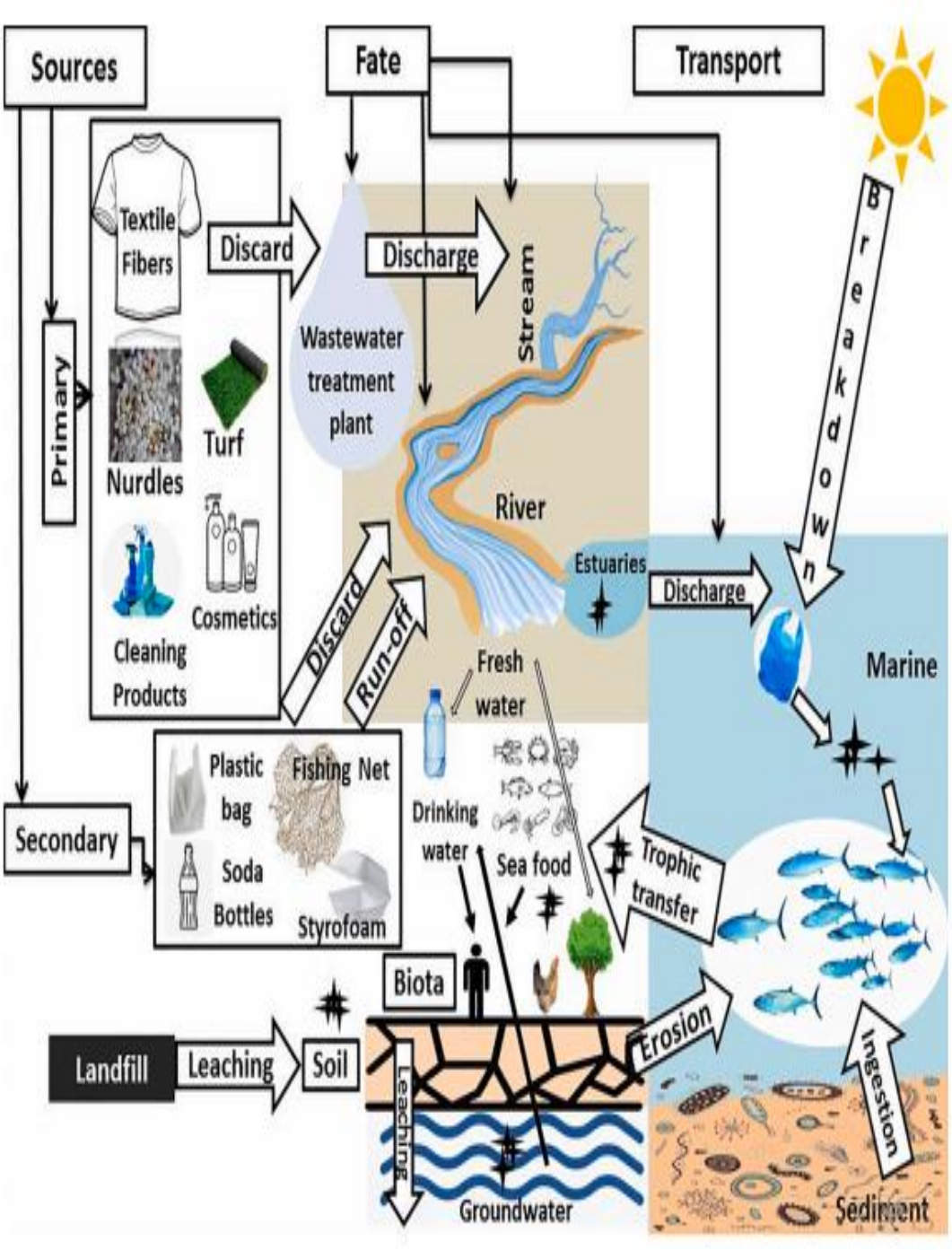


Figure 1.3: Microplastics transfer pathway in aquatic and marine environment

Zooplankton are important food sources for many secondary producers, including commercially important cetaceans and fish (Lee et al., 2013). Zooplankton filter a large number of surface waters contaminated with microplastics for feeding purposes, increasing the risks of microplastic consumption and encounter for higher trophic level species such as fish (Cózar et al., 2014). They can also be consumed by other zooplankton.

1.6. *Artemia salina* (Brine shrimp)

In recent years, aquatic invertebrates have been used to assess the potential toxicity of microplastics. Brine shrimp (*Artemia salina*) plays an important role in the energy flow of the food chain in various seawater systems ranging from lakes to oceans. Because brine shrimp filter a large amount of water per hour, it is referred to as a nonselective filter feeder. As a result, it is more likely than other aquatic organisms to be exposed to pollutants.

Artemia salina undergoes a unique and adaptable life cycle. It begins with the cyst stage, where hardy dormant eggs can endure harsh environmental conditions for extended periods. When conditions become favorable, cysts hatch into nauplii, the earliest larval stage characterized by simple, shrimp-like structures. Nauplii subsequently molts and develop into meta-nauplii, gaining more complexity with features like compound eyes and branched appendages. The life cycle continues through several additional developmental stages, including juvenile and adult stages, depending on environmental cues such as salinity, temperature, and food availability. This adaptability allows *Artemia salina* to thrive in a wide range of aquatic environments and contributes to its importance as a model organism in aquatic research and aquaculture (Nunes et al., 2006).

Artemia salina nauplii are preferred for research over juveniles because they are cultured in only 1-2 days and they are a staple in aquaculture as live feed for fish and shrimp larvae. Their nutritional value and small size make them essential in early stage rearing of aquatic species, making them a valuable model organism for conducting experiments related to nutrition, toxicity, and ecotoxicology (Rajabi et al., 2015).

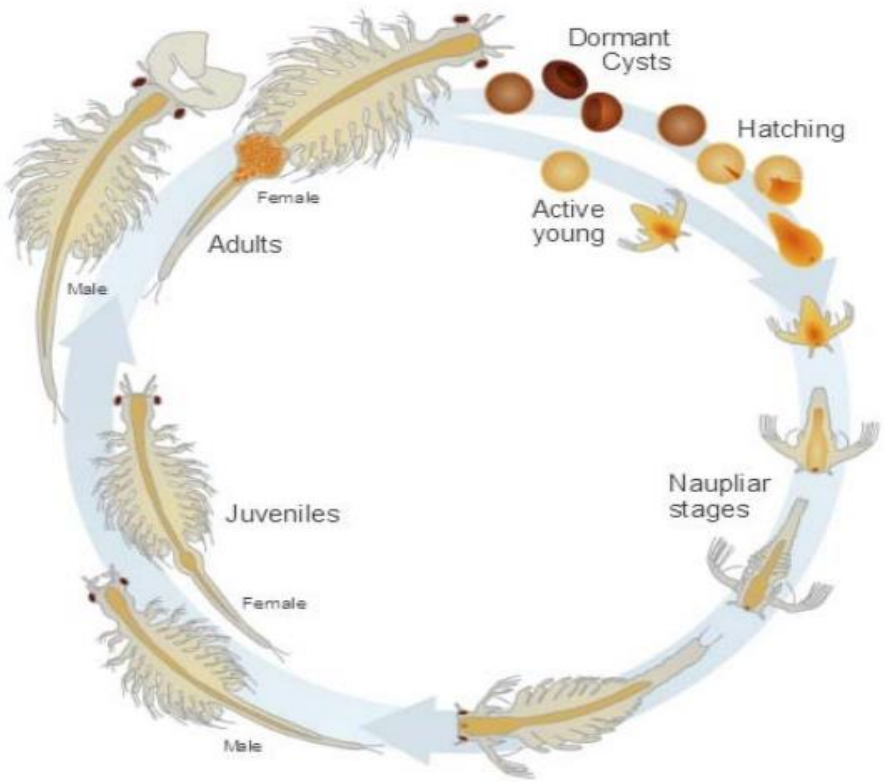


Figure 1.4: Lifecycle of Artemia salina (Nunes et al., 2006)

Many studies have done in past on the effects of microplastics on zooplankton. Jeyavani et al. (2022) reported the effects of polypropylene microplastics on growth, survivability, and swimming activity on different stages of brine shrimp. Suman et al. (2020) studied the adverse effects of polystyrene microplastics on brine shrimps. In another study by Kokalj et al. (2021), effect of virgin and recycled LDPE exposure to Daphnia magna were studied and the results showed that virgin LDPE was more harmful.

1.5. Objectives of study

The objective of the study is as follows:

1. Hatching & growth of Artemia salina at optium conditions.
2. Prepare PP, LDPE and HDPE microplastics and investigate the effect of microplastics on nauplii of *Artemia salina.*
3. To analyses the presence/concentration of microplastics in brine shrimp's body parts through fluorescence spectroscopy.

2. LITERATURE REVIEW

2.5. Micro plastics in Marine ecosystem

Eriksson and Burton (2003) explored the role of microplastics in marine sediments. Their research revealed that microplastics were prevalent in sediment samples collected from different coastal areas, indicating their widespread deposition. The study also highlighted the role of storm events and coastal processes in redistributing microplastics, further contributing to their distribution in marine ecosystems. This work underscored the need for a comprehensive understanding of how microplastics interact with sediments and potentially affect benthic ecosystems (Eriksson & Burton, 2003).

In a study by Thompson et al. (2004), the researchers conducted one of the pioneering investigations into microplastics in the marine environment. They found that microplastics, originating primarily from the breakdown of larger plastic items, were present in marine waters across the globe. These microplastics were identified in various sizes and types, raising concerns about their widespread distribution. The study emphasized the potential consequences of microplastics for marine organisms, as they can be ingested and accumulate in the gastrointestinal tracts of species such as filter-feeding organisms and fish.

Law et al. (2010) explored the interactions between microplastics and marine biota in coastal environments. Their study focused on the ingestion and retention of microplastics by marine worms, which play essential roles in sedimentary ecosystems. The research demonstrated that microplastics can be readily ingested by these organisms, potentially affecting their behavior and ecosystem functions. This work highlighted the intricate relationships between microplastics and benthic communities, suggesting broader ecological implications for coastal ecosystems.

Wright et al. (2013) conducted a study focusing on microplastics in the gastrointestinal tracts of marine birds. Their research revealed that seabirds, such as albatrosses and

fulmars, frequently ingested plastic particles, including microplastics. These particles often led to blockages and digestive issues, posing a significant threat to the health and survival of these birds. The study emphasized the role of marine plastic pollution, including microplastics, in impacting avian populations in coastal and oceanic environments.

Cózar et al. (2014) investigated the abundance and distribution of microplastics in the surface waters of the world's oceans. They found that microplastics were widespread in all sampled oceanic regions, including subtropical gyres and polar waters. The study highlighted the role of oceanic currents in concentrating and redistributing microplastics, leading to their accumulation in specific areas. This research underscored the need for global efforts to mitigate microplastic pollution and its far-reaching environmental consequence.

A more recent study by Lusher et al. (2017) investigated the ingestion of microplastics by marine megafauna, including filter-feeding species like manta rays and whale sharks. Their findings showed that these large oceanic species were exposed to microplastics through their filter-feeding activities, highlighting the presence of microplastics even in remote oceanic habitats. This study emphasized the potential transfer of microplastics up the marine food web, as these large animals are part of a complex trophic network.

Another study focused on a complex trajectory wherein microplastics, originating from various sources, accumulate in marine ecosystems, where they are ingested by small aquatic organisms. These microplastics then ascend through the food web, becoming increasingly concentrated and posing risks to higher trophic levels, including commercially harvested seafood. Consequently, these contaminated marine species

serve as vectors for microplastics' entry into the human food supply, potentially exposing humans to the adverse effects of these ubiquitous pollutants (Du et al., 2021).

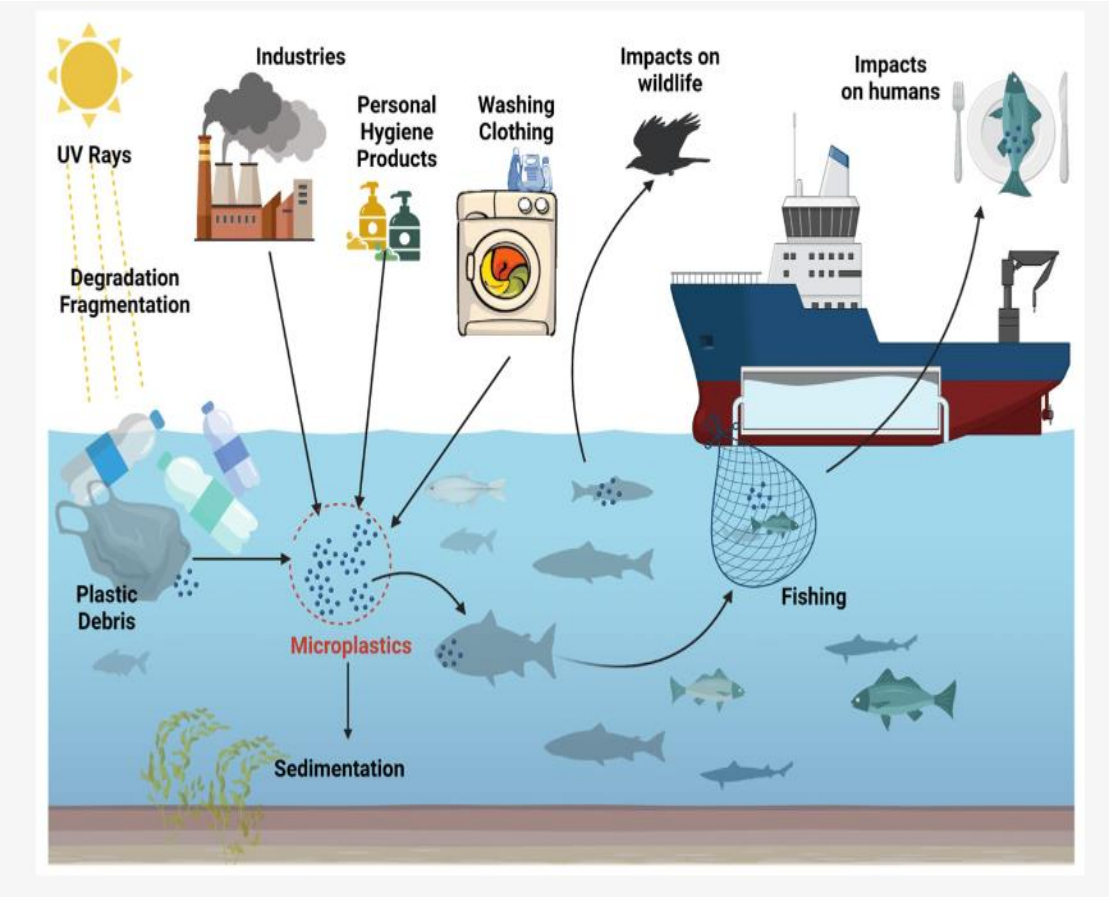


Figure 2.1: Microplastic transfer through marine environment

2.6. Microplastics and zooplankton

Zooplankton are a diverse group of small aquatic animals that play a crucial role in marine food webs, serving as a primary food source for a variety of larger organisms, including fish, whales, and sea birds. They are also important for nutrient cycling and carbon sequestration in marine ecosystems. However, the impact of microplastics on zooplankton is not fully known, and there is growing concern about the potential negative effects of these particles on these important organisms.

In a study by Cole and his coworkers in 2013, researchers examined the interactions between microplastics and zooplankton. They found that zooplankton were prone to ingesting microplastics, particularly microbeads, which affected their feeding rates and overall fitness. Microplastics also acted as vectors for the transport of harmful chemicals, potentially increasing the exposure of zooplankton to toxins. These findings underscored the potential consequences of microplastic pollution for essential components of aquatic food webs.

Jemec et al. (2016) exposed Daphnia magna to different concentrations of polystyrene microbeads, which are a common type of microplastic found in the environment. They found that microplastics reduced the survival of Daphnia magna in a dose-dependent manner. Similarly, a study by Cole et al. (2013) found that microplastics reduced the survival of copepods, another type of zooplankton.

In a field study conducted in 2017, the researchers investigated the prevalence of microplastics in the natural diet of marine copepods. They found that copepods in the field ingested microplastics as part of their natural diet, demonstrating the real-world relevance of microplastics exposure. This study highlighted the potential for microplastics to enter marine food webs through zooplankton ingestion, potentially affecting larger organisms (Jeong et al., 2017).

Welden and his colleagues in 2018 examined the behavioral responses of zooplankton to microplastics exposure. They observed that zooplankton exhibited altered swimming behaviors and reduced foraging efficiency when exposed to microplastics. These changes could have cascading effects on their survival and trophic interactions within aquatic ecosystems. The study emphasized the importance of understanding the sublethal effects of microplastics on zooplankton (Welden et al., 2018).

Grigorakis and coworkers (2017) studied the gut retention of plastic microbeads and microfibers in goldfish (Carassius auratus) was investigated. The primary focus was on assessing the duration of time these microplastics persisted within the digestive system of the fish. The research findings revealed that both microbeads and microfibers were retained in the goldfish's gut for an extended period.

Zebrowski and his coworkers investigated the ingestion and effects of microplastics on daphnia, a common freshwater zooplankton species. They found that microplastics were readily ingested by daphnia, leading to reduced feeding rates and increased mortality. Moreover, microplastics affected daphnia's reproductive output. This study provided insights into the potential population-level impacts of microplastics on zooplankton species (Zebrowski et al., 2022).

A study examined the interaction between microplastics and marine copepods. They discovered that microplastic ingestion caused oxidative stress in copepods, leading to cellular damage and reduced survival rates. This research highlighted the physiological stress that zooplankton can experience when exposed to microplastics, which may have implications for their ecological roles in marine ecosystems (Vroom et al., 2020).

Hossain et al. (2020) conducted a study to investigate the effects of microplastics on the growth and reproduction of copepods, a crucial group of zooplankton. They found that exposure to microplastics led to reduced reproductive success, delayed development, and decreased body size in copepods. These impacts had the potential to ripple through the food web, affecting higher trophic levels. The study highlighted the vulnerability of zooplankton to microplastic-induced stress.

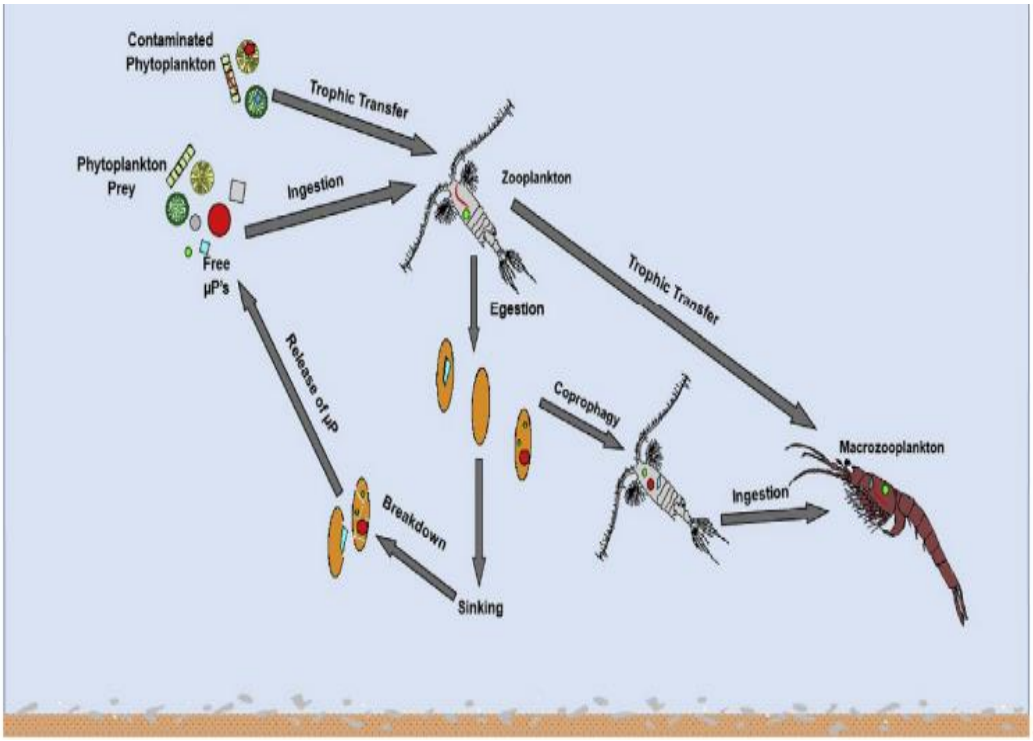


Figure 2.2: Schematic diagram of microplastic uptake by zooplankton

2.7. Effect of microplastics on *Brine shrimp*

In a study conducted in 2019, the researchers investigated the uptake and elimination of polystyrene microplastics by the brine shrimp, *Artemia parthenogenetica,* and explored how this exposure influenced the shrimp's feeding behavior and intestinal histology. The main findings revealed that the brine shrimp readily ingested microplastics, leading to alterations in their feeding patterns characterized by reduced feeding rates. Furthermore, examination of the shrimp's intestinal tissues showed signs of inflammation and damage caused by the presence of microplastics. These results highlight the potential adverse effects of microplastic pollution on aquatic organisms, emphasizing the need for further research and environmental mitigation efforts to address this growing ecological concern (Wang et al., 2019).

Another study in 2019 investigated the uptake and consequences of various concentrations of spherical polymer microparticles on *Artemia franciscana*. The primary objective was to assess how these microplastic particles affected the brine shrimp, both in terms of ingestion and potential physiological repercussions. The main findings revealed a concentration-dependent uptake of microparticles by *Artemia franciscana,* with higher concentrations resulting in increased ingestion rates. Additionally, exposure to elevated concentrations of microparticles led to adverse effects on the survival and growth of the brine shrimp, indicating the potential for ecological repercussions in marine ecosystems (Peixoto et al., 2019).

Nausheen and coworkers in 2022 investigated the toxic effects of both pristine and aged polystyrene microplastics on the selective and continuous larval culture of the acorn barnacle, Amphibalanus amphitrite. Researchers examined how exposure to these microplastics impacted the survival and development of barnacle larvae. The main findings revealed that while both pristine and aged microplastics had detrimental effects on larval survival, aged microplastics were particularly toxic, leading to reduced larval settlement and metamorphosis success rates. This underscores the increased ecological risk posed by weathered microplastics in marine environments and emphasizes the

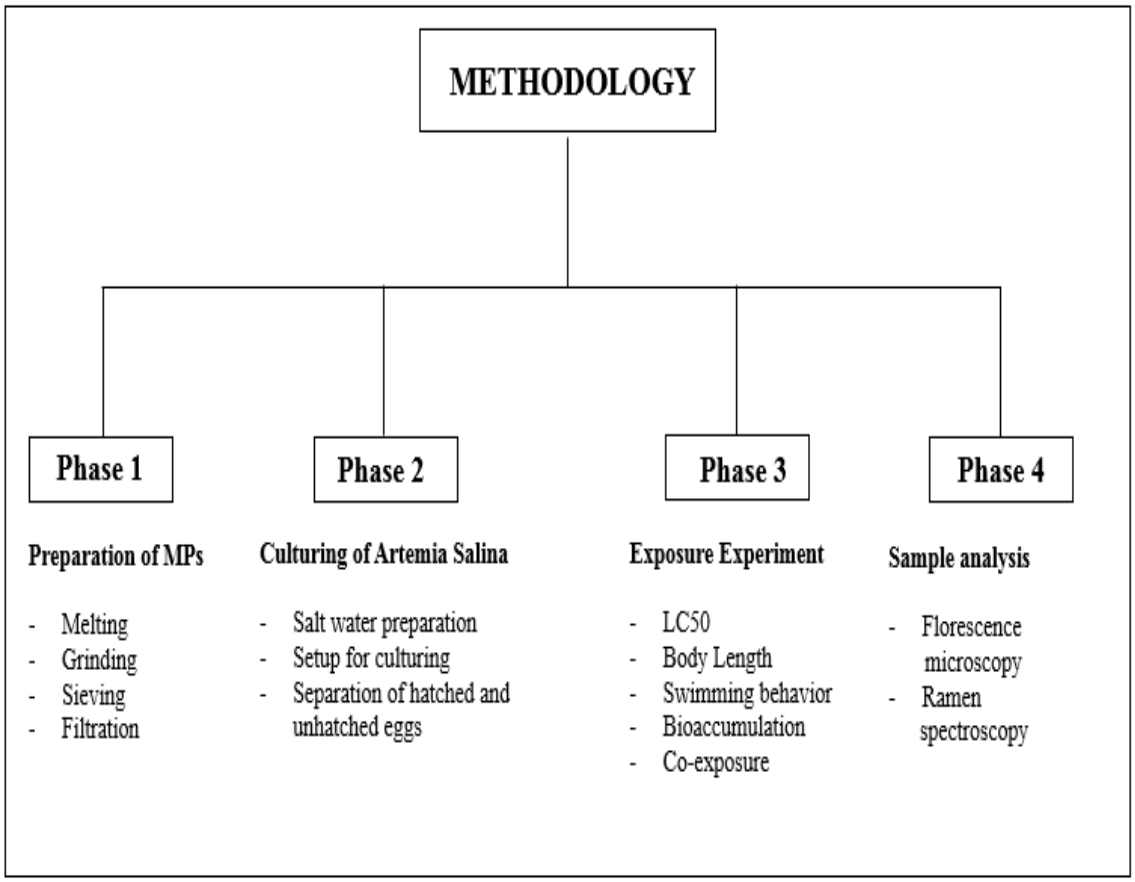
urgent need for mitigation measures to address the harmful consequences of microplastic pollution on marine organisms (Nousheen et al., 2022).

Jeyavani and coworkers in 2022 examined that upon exposure to these microplastics, the survival rates of the marine microcrustacean were significantly reduced, indicating a heightened vulnerability to this type of pollution. Additionally, the behavioral responses of *Artemia salina* were disrupted, suggesting potential disturbances in their ecological interactions and predator avoidance mechanisms. Moreover, the growth and development of the organisms were hindered, which could have cascading effects on the entire food web. The study's most notable revelation, however, was the potential for bioaccumulation of polypropylene microplastics in the digestive tract of *Artemia salina.*

These studies collectively demonstrate that *Artemia salina* is susceptible to the ingestion of microplastics, which can lead to a range of adverse effects, including altered behavior, reduced growth, changes in reproduction, and physiological stress. These findings underscore the importance of understanding the ecological consequences of microplastic pollution for key zooplankton species in aquatic ecosystems.

3. METHODOLOGY

The methodology adopted for the study was divided into four phases as described in figure below:



3.1. Preparation of MPs

For the preparation of micro-plastics, plastic beads of polypropylene (PP), low-density polyethylene (LDPE) and high-density polyethylene (HDPE) that are used in procedures like injection molding procedures were purchased from a vendor. These plastic beads were then washed with ethanol to remove all the impurities. They were then dried at room temperature and stored in an airtight container.

(a) (b)

(c)

Figure 3. 1: Pictures of virgin plastics beads of (a), HDPE , (b) LDPE, and (c) PP

3.1.1. Melting plastics

Plastic beads were placed inside a glass petri dish, and then, a carefully controlled heating process was initiated using a hotplate, targeting the specific melting points of each plastic type. For instance, the polypropylene beads were subjected to a temperature of approximately 160°C for a duration of roughly 6-8 minutes. Similarly, the LDPE (Low-Density Polyethylene) and HDPE (High-Density Polyethylene) beads were melted at temperatures of 110°C and 125°C, respectively. The primary objective behind melting these plastic beads was to transform them into a molten state, allowing them to be shaped and molded into larger pallets, which would facilitate easier handling during subsequent manufacturing processes. Once the desired shapes were achieved through this heating and molding process, the newly formed plastic structures were then left to cool naturally at room temperature. This cooling step is essential to ensure that the plastic materials solidify and retain their intended shapes for their intended applications.

Figure 3.2: Flow diagram of microplastics melting procedure

3.1.2. Grinding of Plastics

In the subsequent phase of the process, the transformation of plastic pallets into finely grained plastic powder was undertaken. This step departed from conventional methods that relied on silicon carbide paper, as documented by Rodríguez Chialanza et al. (2018), Instead, an innovative approach was adopted, utilizing a nail filler constructed from stainless steel. This nail filler proved to be an effective alternative, demonstrating its capacity to efficiently grind the plastic pallets into a powdered form. This finely grained plastic powder holds significant promise in various applications, including recycling and material processing, where the uniformity and consistency of the particle size are crucial. The adoption of stainless-steel nail fillers represents a noteworthy advancement in the field, offering a more sustainable and efficient means of plastic waste reduction and repurposing. The nail filler made of stainless steel of was used. The nail filler converted the plastic pallets into finely grained plastic powder.

Figure 3.3: Nail filer used for crushing plastic pallets

Figure 3.4: Powdered microplastics in petri dish

3.1.3. Sieving and filtration

After crushing the plastic pallets, the microplastics of varying sizes were dispersed in the distilled water and subjected to sonication for approximately 15 to 20 minutes. Following sonication, the solution containing microplastics underwent a filtration process using vacuum filtration. To achieve the desired microplastic sizes, two different (*American Standard Test Sieve Series (ASTM) - Endecotts*, n.d.). were employed, specifically the 625 and 450 numbered sieves, which corresponded to particle sizes of 20 and 32 microns, respectively. These sieves were utilized in place of traditional filter paper within the filtration assembly.

During filtration, particles larger than the respective sieve sizes (20µm and 32µm) remained on top of the sieves, while smaller microplastics were successfully collected in the filtrate. Subsequently, the filtrate underwent an additional filtration step using Whatman filter paper with a pore size of 0.45 microns. This meticulous filtration

process allowed for the isolation of microplastics falling within the size range of 0.45µm to 20µm, which were then collected on the filter paper. Finally, to facilitate further analysis, the filter paper was carefully dried at room temperature, and the collected microplastics were gently brushed into Eppendorf tubes for subsequent examination and characterization. This systematic approach ensured the precise isolation and collection of microplastics within the specified size range, crucial for accurate analysis and research findings in the study.

(a) (b)

Figure 3.5: Microplastics solution and filtration assembly used for filtration

3.1.4. Particle Size analysis

A Horiba LA-300 Particle Size Analyzer was employed to assess the particle size distribution of microplastics in a wet solution containing a surfactant. This advanced instrument boasts a wide measurement range spanning from 0.1 to 600 microns, making it suitable for the precise analysis of particles falling within this size spectrum (Gola et al., 2021). To ensure accurate and reliable results, several meticulous steps were taken. Prior to particle size analysis, the sample underwent sonication and circulation to disperse and suspend the microplastics uniformly in the solution. The Horiba LA-300 operates on the principle of Dynamic Light Scattering (DLS), a method that has been established by previous research, as highlighted by Wang et al. (2020), as effective for quantifying microplastics. Additionally, meticulous precautions were taken, such as chamber rinsing to prevent cross-contamination and the removal of any bubbles in the sample to eliminate interference. A blank test was conducted to align the laser, ensuring the accuracy of measurements, and subsequently, mean, mode, and median values were calculated to provide a comprehensive understanding of the microplastics size distribution in the analyzed samples. 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.

Figure 3. 6: Horriba LA-300, Particle size analyzer

3.1.5. Raman spectroscopy analysis

The powdered microplastics samples underwent thorough analysis using Raman spectroscopy. This analytical technique, employing a Bio-Rad FTS 175C instrument equipped with a Raman accessory, enabled the assessment of both sample purity and chemical properties (Karami et al., 2017). The analysis was carried out using FT-Raman (Fourier-transform Raman) spectroscopy, with a wavelength of 1064nm. FT-Raman spectroscopy is particularly valuable for its ability to provide detailed information about molecular composition and structural characteristics of the examined material (Renner et al., 2018).

Figure 3.7: Raman spectroscope used for confirmation tests of microplastics

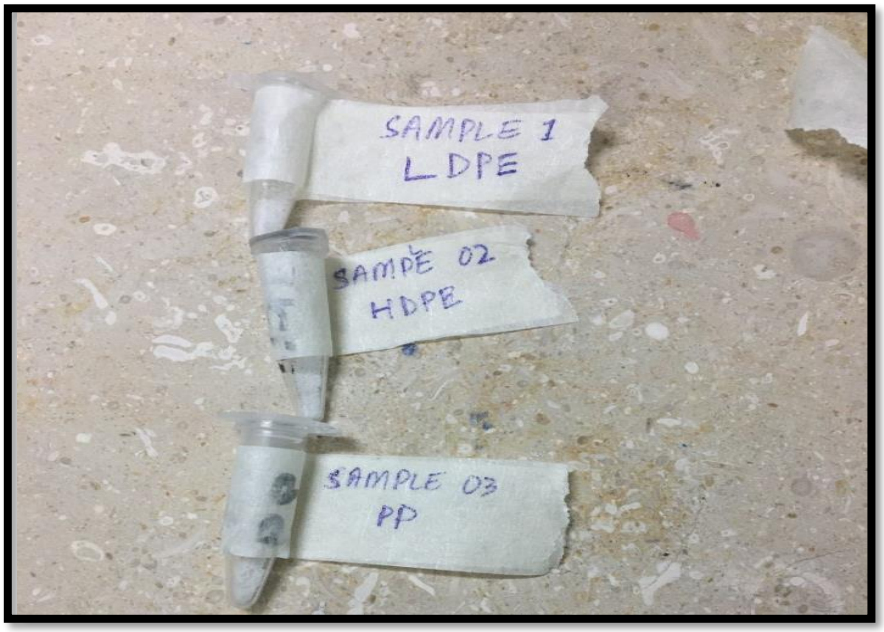


Figure 3.8: Powdered microplastics of size <32 subjected to Raman spectroscopy analysis

3.2. Culturing of *Artemia Salina:*

Artemia salina cysts were sourced from Daraz.pk, a well-known online retail platform. To maintain their viability and ensure their longevity, the cysts were carefully stored in an airtight jar at a temperature of 4°C. This controlled storage environment is crucial for preserving the dormant state of the cysts until they are needed for hatching and experimentation.

Figure 3.9: Artemia salina Cysts

3.2.1. Preparation of saltwater

Salt water was prepared by adding 25 g of NaCl in one liter of water. The physical parameters such as temperature, pH was also maintained according to the reported values (Jeyavani et al., 2022). Temperature was maintained at 28ºC by using aquarium heating rod whereas pH of the water was recorded between 7.1- 8.9. The aquarium aerator was used for continuous aeration of water and a light bulb was set-up for illumination as shown in figure 3.8. Half teaspoon of brine shrimp cysts was added in the salt water for hatching. Under continuous aeration and illumination, the brine shrimp cysts took 18-24 hours to hatch as reported by Gambardella et al., (2017).

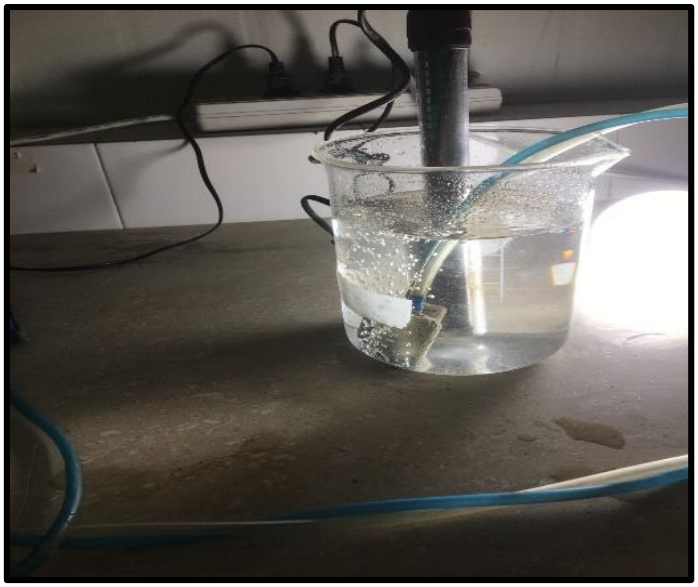


Figure 3.10: Saltwater set-up for Artemia salina hatching

3.2.2. Separation of hatched and unhatched *Artemia salina* eggs

After 24 hours, the aerator was turned off so that the hatched *Artemia salina* can be separated from unhatched ones. The hatched *Artemia salina* eggs were floating on the water surface whereas unhatched eggs settled at the bottom. The hatched stage I *Artemia salina* known as instar I were gathered near the light source. They were then transferred to a clean beaker having salt water via dropper.

3.2.3. Feeding of *Artemia salina*

During the Instar I larval stage, these organisms do not actively consume food. Feeding initiation occurs at the subsequent Instar II larval stage. To provide nourishment at this crucial stage, a commercial fish food was prepared by grinding it into a fine powder using a pestle and mortar. This powdered fish food was then introduced to the *Artemia salina* nauplii. Feeding was administered judiciously, with a pinch of the powdered food being offered to the nauplii every two days. This gradual introduction of food ensures that the *Artemia salina* receive the necessary nutrients to support their growth and development as they transition into the next developmental stage as reported by Sussarellu et al., (2016).

Figure 3.11: Powdered spirulina fish food used as a feed for Artemia salina

3.3. Exposure of MPs to *Artemia Salina*

Two series of tests were performed: (a) mono exposure of microplastics (b) co-exposure of microplastics. Both experiments were run in triplicates and were compared with blank.

3.5.1: Mono exposure of microplastics

In the first series of tests, 5 suspensions of each microplastics type having 150 mg/l the highest were made.1000 mg/l stock solution was prepared using artificial seawater for PP, LDPE and HDPE separately. The stock solution was sonicated for 20 minutes and working concentrations of 25, 50,75,100 and 120 mg/l were prepared from that stock solution as reported by previous researchers (Jeyavani et al., 2022; Suman et al., 2020).

3.5.2 Coexposure of microplastics.

In second series of tests, a mixed suspension of all three microplastics was prepared. For co-exposure, 1000 mg/l stock solution of 33 % each microplastics was made and sonicated for 20 minutes. From that, working concentrations of 25,50,75, 100 and 125 mg/l were made.

3.5.3 24 h toxicity exposure

To determine the ingestion of microplastics by *Artemia salina* and its effects on organism's survival, *Artemia salina* were exposed to microplastic concentration. In both a and b experiments, 20 *Artemia salina* nauplii were added in each microplastics concentration (100 *Artemia salina* nauplii in each experiment altogether) and were exposed to microplastics for 24 h in triplicates.

Figure 3.12: Exposure setup in triplicates for mono and coexposure of microplastics

After 24 h, a series of tests were performed to find out the effects of microplastics on following Parameters:

1. LC50 assessment
2. Bioaccumulation assessment
3. Body length
4. Swimming behavior

3.3.1. LC50 assessment of MPs

Lethal concentration is the value of MPs concentration at which half of the population of brine shrimps die. For the calculation of L550, five concentrations were made from the stock solution for each MPs.

a) LC50 setup for mono and co-exposure of microplastics

As described earlier, five concentrations such as 25 mg/l, 50 mg/l, 75 mg/l ,100 mg/l and 125 mg/l were prepared from stock solution of 1000 mg/l for both series of tests (Suman et al., 2020). A total of 20 brine shrimps nauplii were added in each concentration. After 24 h the number of dead organisms were counted. The organisms that were immobile for more than 10 seconds were also considered dead.

3.3.2. Bioaccumulation assessment

To check whether the Microplastics bio-accumulate in the body of brine shrimps or not, brine shrimps were exposed to 25,50,75 ,100 and 125 mg/l concentrations of PP, LDPE and HDPE separately and in combination (Grigorakis et al., 2017). Microplastics of the three types were fluorescently labelled before the preparations of concentrations.

a) Nile Red staining

Microplastics were fluorescently labelled with the help of a renowned dye named **Nile red**. Nile red solution was made by dissolving 1 mg of Nile red dye powder into 5 ml ethanol (Pan et al., 2022) and was placed in a dark area for almost 30 minutes. After 30 minutes the dye was sprayed onto the plastics with the help of syringe and allowed the plastics to dry at room temperature.

Figure 3.13: Nile Red dye used for staining microplastics

b) Removal of excess dye from microplastics

After drying the dyed microplastics, they were washed with distilled water three times so that the excess dye is removed from the microplastics. After that they were dried at room temperature and stored for their usage in further analysis.

c) Bioaccumulation experiment

The fluorescently labelled microplastics of three different types were used to make five separate concentrations of 25,50,75, 100 and 125 mg/l from both mono and coexposure. A Total of 20 brine shrimp nauplii were added in each concentration of both experiments. After 24 h exposure, the brine shrimps were cleaned with deionized water three times and were analyzed under fluorescent microscope (Optica B-52) to check the bioaccumulation of microplastics in the gut of brine shrimp (Wang et al., 2020).

Figure 3.14: Optics B-52 Fluorescent microscope

d) Calculation of number of particles per organism

To calculate the number of particles per organism, following steps were followed:

i) Digestion of *Artemia salina*

In the quantitative determination of microplastic particle ingestion by brine shrimps (*Artemia salina*), an adapted methodology was implemented with precision. For each concentration of LDPE (low-density polyethylene), 20 individual brine shrimp specimens were carefully selected and placed into separate test tubes containing a solution comprising 69% nitric acid. This nitric acid solution served as a potent digestive agent, facilitating the degradation of organic matter within the brine shrimps.

Subsequently, these test tubes, each containing a set of 20 brine shrimps, underwent a controlled digestion process. The digestion was carried out under rigorous temperature control, with the samples being subjected to a temperature of 70 ^{O}C for an extended period of 3 hours. This deliberate duration was chosen to ensure the comprehensive breakdown of organic components, leaving behind only the microplastic particles that the brine shrimps had potentially ingested (Fortin et al., 2019) .

Following the 3-hour digestion period, the solutions within the test tubes were meticulously diluted with distilled water. This dilution step was imperative to optimize the analytical concentration of the solution for subsequent quantification procedures.

Subsequent to dilution, the solutions were subjected to filtration employing glass fiber filter papers possessing a fine pore size of 0.2µm. This filtration step was instrumental in effecting the separation of microplastic particles from the solution matrix, thus facilitating their isolation and collection on the filter paper substrate. The same procedure was followed for HDPE, PP and co-exposure concentrations.

ii) Calculation of number of particles in mono exposure

The microplastic particles on dried filter papers were then analyzed using DSX Olympus digital microscope. The particles in 20 *Artemia salina* were counted on each filter paper and from that data number of particles per organism were calculated.

iii) Calculation of number of particles in co-exposure

For co-exposure experiment, the filter papers were first examined by DSX Olympus digital microscope and after that, to check which microplastics was ingested more by *Artemia salina* , the filter papers were processed by Raman spectroscopy (Karami et al., 2017).

Figure 3. 15: DSX , Olympus Digital Microscope

3.3.3. Body length

To check whether ingestion of microplastics has any influence on body length of brine shrimps, the total body length of brine shrimps from control group and from exposure groups was measured. For that *Artemia salina* were exposed to 25 mg/l microplastics concentrations for 72 hours (3 days) in both exposure experiments. Olympus digital microscope of model DSX1000 was used. The body length of shrimps was measured from head to anus. The exposure period was chosen to mimic a realistic scenario of microplastic interaction with these aquatic organism (Li et al., 2021).

3.3.4. Swimming behavior

In order to investigate potential alterations in the swimming behavior of brine shrimp resulting from the ingestion of microplastics, an examination involving brine shrimp specimens that had been exposed to various concentrations of microplastics individually (specifically, LDPE, HDPE, and PP), as well as through coexposure experiments was conducted.

To scrutinize their swimming patterns and behaviors, brine shrimp samples from each of these microplastic exposure scenarios were collected and were then carefully observed and recorded using a smartphone, capturing 1-minute-long videos of their swimming activities. Subsequently, a specialized android application was employed called "Animap"(Rao et al., 2019).

This Animap application proved to be a valuable tool in research, as it not only recorded the swimming behaviors of the *Artemia salina*, but it also transformed this visual data into informative graphical representations. These graphical outputs allowed us to analyze and compare the swimming patterns of the brine shrimp under different microplastic exposure conditions, shedding light on any potential effects of microplastic ingestion on their locomotion.

4. RESULTS AND DISCUSSIONS

4.1. Microplastics characterization

The size of microplastics determined by Particle Size Analyzer (Horriba LA-300) was between 32 – 0.2µm as shown in table 4.1 and figure 4.1. Based on the data depicted in Figure 1, it is evident that PP sample encompasses microplastic fragments spanning a size range of 1.3 to 6.7µm, exhibiting a maximum particle size of 2.9µm, and displaying an average value of 3.38µm. Whereas LDPE and HDPE samples reveal microplastic fragments spanning a broader size spectrum of 2.9 to 77µm, with maximum particle sizes falling within the range of 15 to 34µm. The mean values for LDPE and HDPE samples are determined to be 24.17 and 24.76µm, respectively. Similar technique was used by (Rostami et al., 2021), who also identified microplastics in personal care product of size ranging from 5 to 483µm using Particle size analyzer. Similarly, another researcher used a focused beam reflectance measurement (FBRM) instrument and a particle size analyzer to measure the sizes of microplastics in water samples. (Primpke et al., 2020). He found that FBRM was an effective method for detecting microplastics smaller than 10µm, while the particle size analyzer was better suited for larger microplastics. A method for detecting and identifying microplastics using Raman spectroscopy and laser-induced breakdown spectroscopy was described where the authors used a particle size analyzer to measure the size distribution of the microplastics in their samples, and found that the majority of particles were in the range of 50-500µm (Cowger et al., 2021). Another study by Eerkes-Medrano et al. (2015) discussed various methods for detecting and measuring microplastics in freshwater systems, including the use of particle size analyzers. The authors note that particle size analyzers can be useful for determining the size distribution of microplastics in water samples.

Table 4.1: Results of Particle size analyser

Sr No	Laser Transmittance%	Median (µm)	Mean (µm)	Variance (µm)	S.D. (µm)	Mode (µm)
PP	99.3	3.38	**3.65**	1.87	1.37	3.19
LDPE	99.5	23.61	**24.17**	100.10	10.00	24.48
HDPE	99.7	24.03	**24.76**	98.90	9.94	24.54

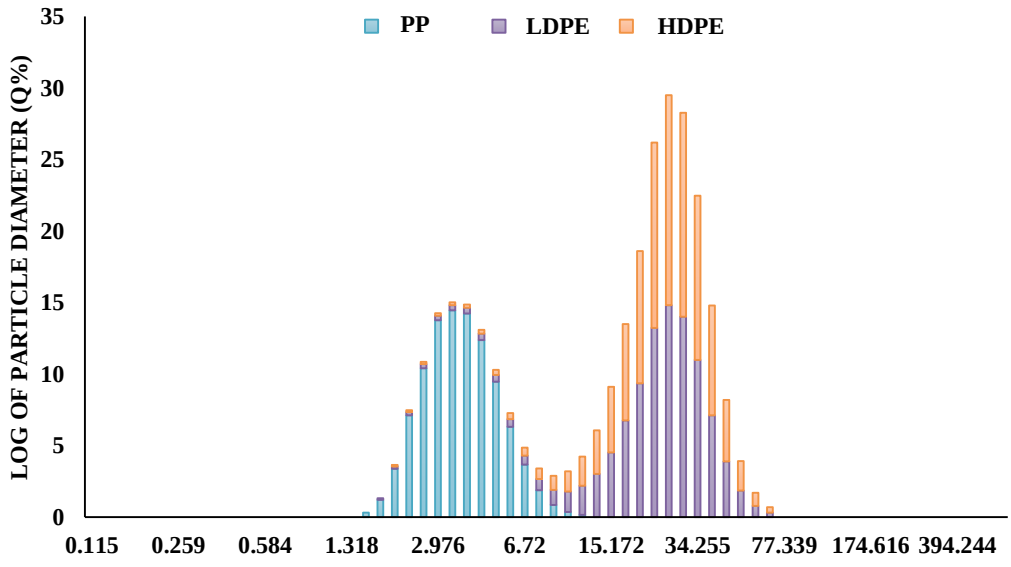


Figure 4.1: Microplastic sizes of PP, LDPE and HDPE samples

4.2. Raman spectroscopy

Raman spectroscopy was used to identify the microplastics samples of PP, LDPE and HDPE as reported by researchers (Araujo et al., 2018; Furukawa et al., 2006) in their studies. The graphs of results of present study also revealed that the samples of LDPE, PP and HDPE have same peaks as present in the standards. Most peaks of PP were in between 100 –1500 cm^{-3} which were also present in the standard PP graph. Similarly, the peaks of LDPE sample were between 1000-1500 cm^{-3} which were same as present in the sample. The HDPE peaks of both sample and standard were also between 1000 – 1500 cm^{-3}. These results also showed the purity of samples. PP was 91 % pure whereas HDPE and LDPE were 93 % and 88 % respectively. The comparison of sample spectra with reference spectra is shown in 4.2, 4.3, 4.4.

Raman spectroscopy was also used in 2018, to identify microplastics in marine sediment and water samples. The study found that Raman spectroscopy was able to accurately identify PP, PE, and PS microplastics in the samples (Holmes et al. 2018).

Fortin and his coworkers in 2019 employed Raman spectroscopy to quantify and identify microplastics in the effluent of advanced wastewater treatment systems. They aimed to assess the effectiveness of these systems in removing microplastics and to characterize the remaining particles. The main findings demonstrated the utility of Raman spectroscopy in precisely identifying microplastic types in the treated effluent, revealing the presence of polyethylene and polypropylene as predominant constituents.

Araújo and his colleagues in 2018 highlighted the ability of this technique to distinguish microplastics from other particles in complex environmental samples, showcasing its applicability in both research and monitoring efforts.

Another study extensively examined the molecular structure, crystallinity, and morphology of Polyethylene (PE) and Polypropylene (PP) blends through Raman spectroscopy. The main findings revealed that Raman spectroscopy offered detailed

insights into the distribution and interaction of PE and PP within the blends (Furukawa et al., 2006).

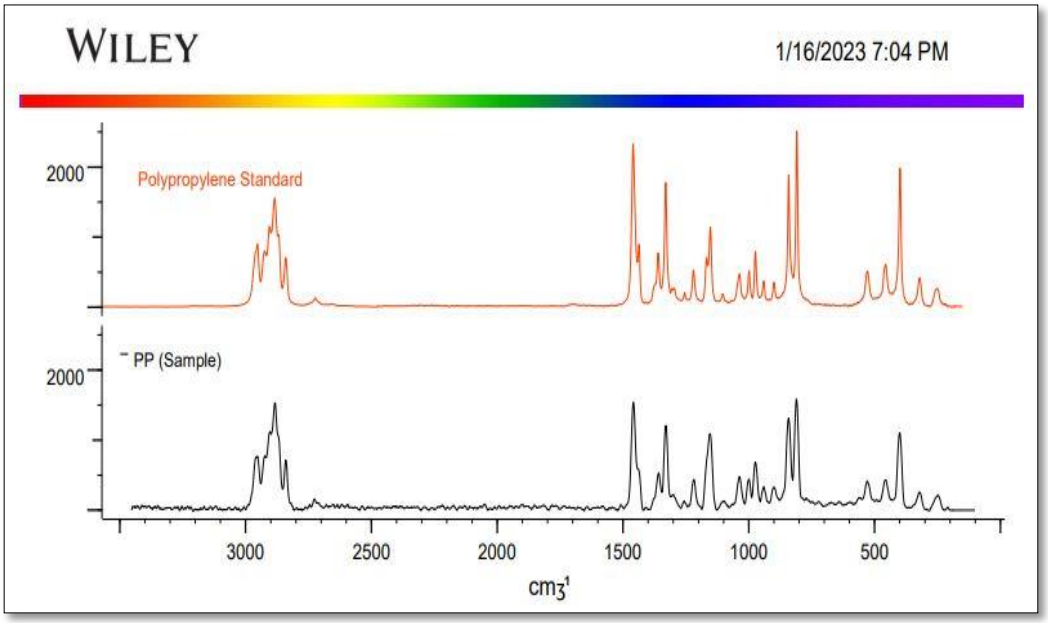


Figure 4.2: Comparison of polypropylene sample with standard

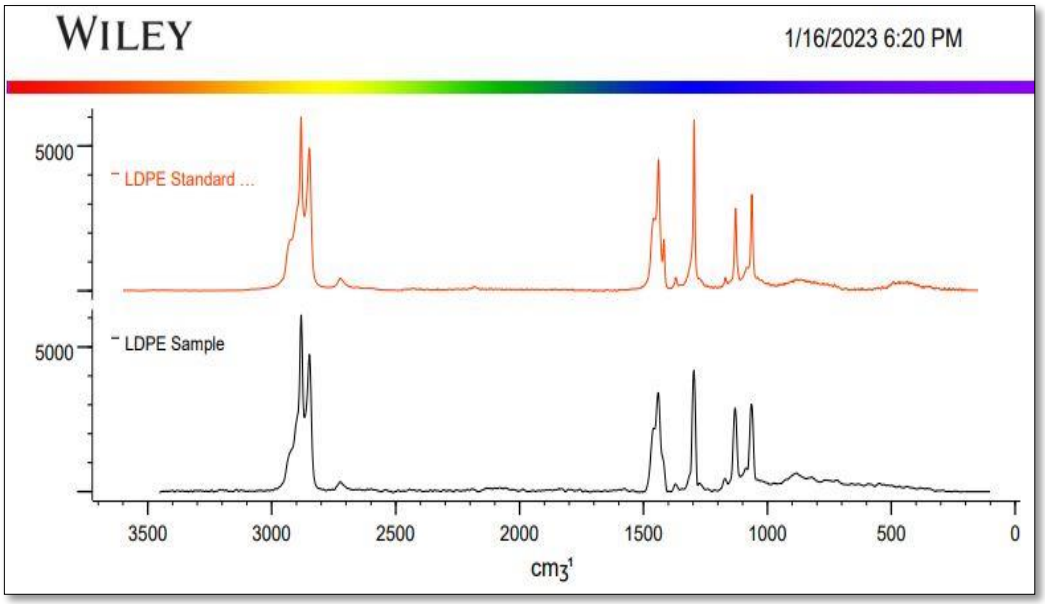


Figure 4.3: Comparison of LDPE sample with standard

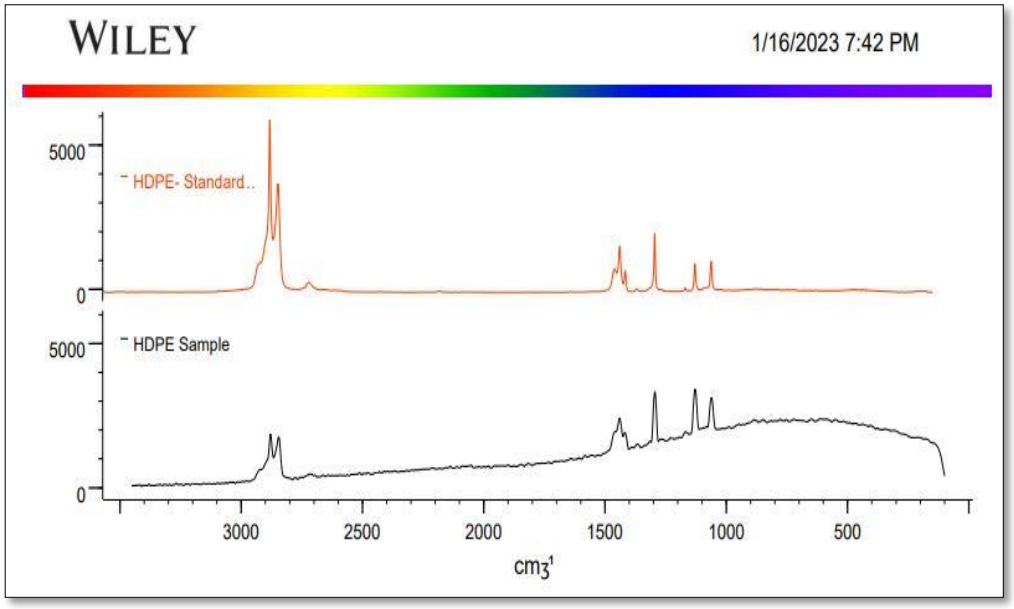


Figure 4.4: Comparison of HDPE sample with standard

4.3. Influence of microplastics ingestion on survival of *Artemia Salina*

4.3.1. LC50 analysis

The LC50 values of mono exposure of microplastics in concentrations ranging from 25mg/l – 125 mg/l were in the order of LDPE > HDPE > PP which revealed that PP is more lethal in the three microplastics. Whereas the LC50 value of coexposure of microplastics was even more low which showed that coexposure of microplastics is more lethal to *Artemia salina* nauplii instead of single microplastic exposure (Table 4.2, Figure 4.5). This showed that the LC50 values are directly proportional to exposure concentrations as reported (Jeyavani et al., 2022). Suman and his coworkers in 2020 also reported that increasing concentration of microplastics results in increasing values of LC50.

Table 4.2: LC50 values of microplastics exposures (mono and coexposure)

Concentration (mg/l)	*Artemia salina* mortality			
	Mono-exposure			Coexposure
	HDPE	LDPE	PP	
0	0	0	0	0
25	1	0	0	3
50	4	3	2	6
75	5	6	4	9
100	10	10	8	13
125	12	12	10	17
LC50	**107**	**103.7**	**124.6**	**68.2**

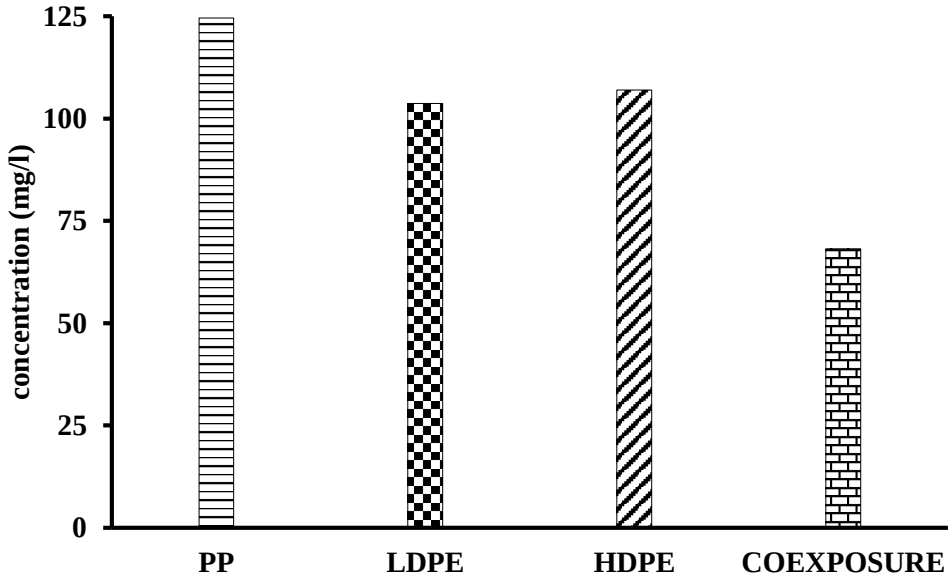


Figure 4.5: Comparison of LC50 values of PP, LDPE, HDPE and coexposure after 24 hours

4.3.2. Mortality rate

Mortality rate in mono exposure of PP, LDPE and HDPE also increased with the increase in concentration (1, 25, 50, 75, 100, 120 mg/l) in the order HDPE >PP>LDPE. Mortality rate in mono exposure of PP, LDPE and HDPE was not very significant in lower concentrations (0-50 mg/l) whereas it increased rapidly in higher concentrations (75-125 mg/l) with a highest mortality rate of 61 % in 125 mg/l in both HDPE and LDPE mono exposures. Whereas in coexposure the mortality rate increased rapidly in both lower and higher concentrations with 15 % recorded at 25 mg/l (lowest concentration) and 80 % at 125 mg/l (highest concentration) (Fig 7,8). Increase in mortality of crustaceans due to microplastic ingestion has been reportedly multiple times in the past decade (Ding et al., 2022; Jemec et al., 2016; Nousheen et al., 2022). Acute exposure to polystyrene nano plastics (0, 5, 25, 50, 100 g/mL) resulted in mortality in *Artemia salina* larvae (instar II) (Bergami et al., 2016). Similar to this, 48-hour acute exposure to polystyrene microbeads with a diameter of 0.1 m resulted in mortality in *Artemia salina* (Gambardella et al., 2017).

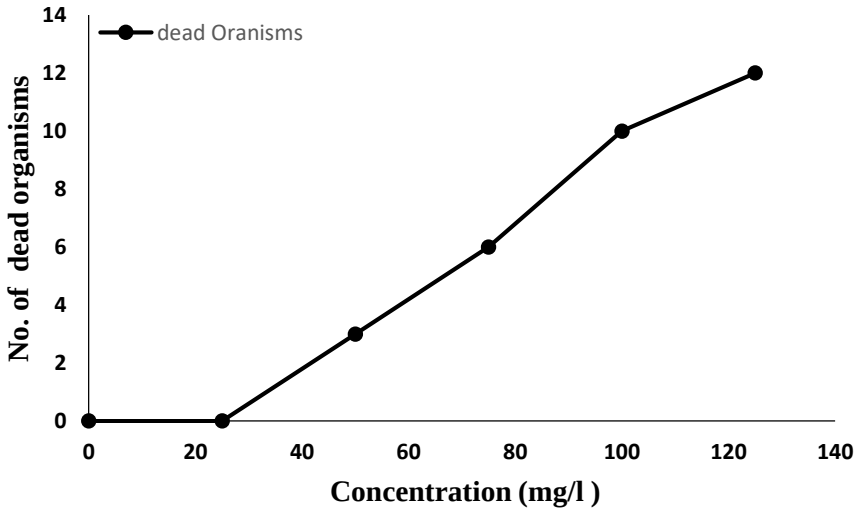


Figure 4.6: Number of dead organisms after 24 h exposure to LDPE

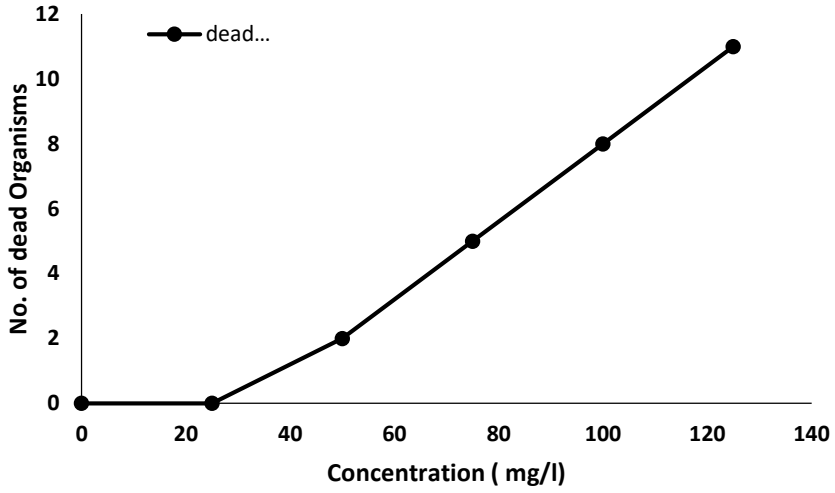


Figure 4.7: Number of dead organisms after 24 h exposure to HDPE concentration

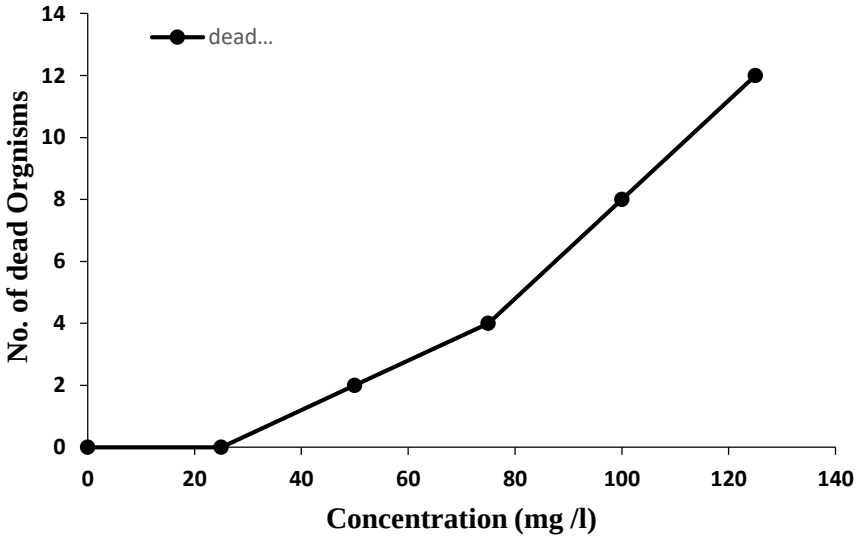


Figure 4.8: Number of dead organisms and mortality rate with increasing concentration

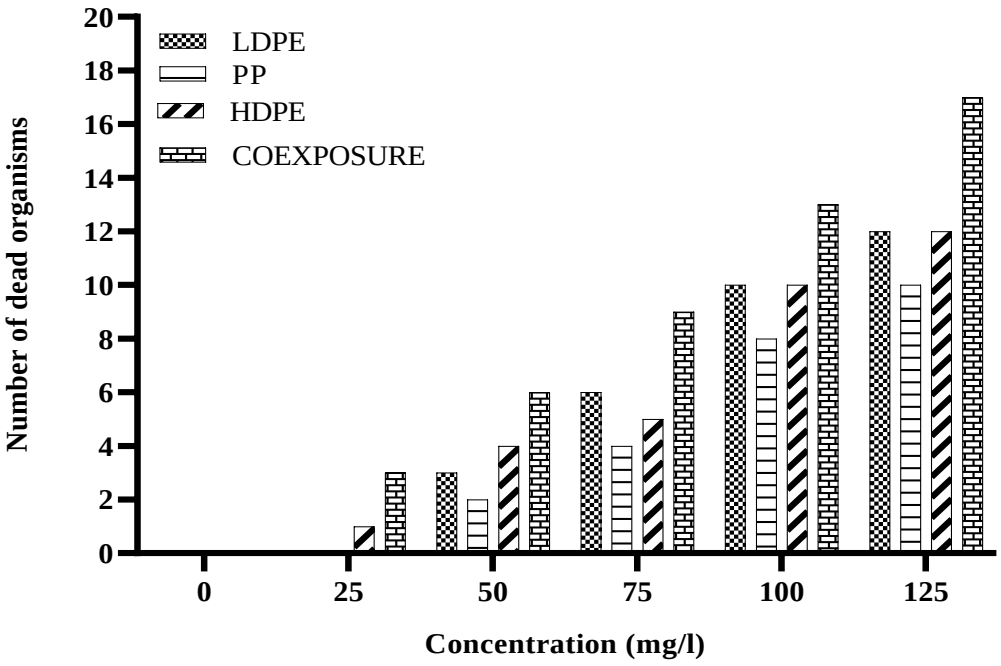


Figure 4.9: Comparison of number of dead organisms in PP, LDPE, HDPE and coexposure

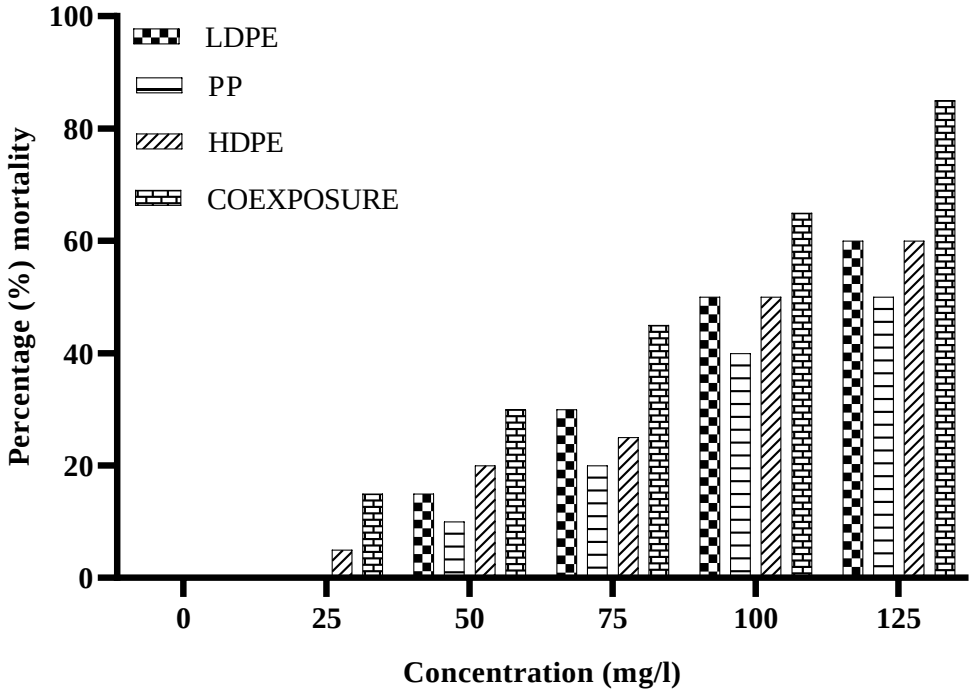


Figure 4.10: Comparison of percentage mortality of LDPE, HDPE, PP and coexposure

4.3.3. Body Length

Results from DSX digital microscope revealed that the body length decreased in the order of HDPE>PP>LDPE which further decreased in coexposure (Table 4.3). A study was conducted in 2021 which indicated that the abundance of polystyrene microspheres significantly affected the body length of *Artemia salina*. This reduction in body length was observed in a dose-dependent manner, with higher microplastic concentrations leading to more pronounced negative impacts on the *Artemia salina's* growth. Another study also revealed that ingestion of microplastics leads to reduction of overall body length of crustaceans because of nutrition deficiency in organism (Li et al., 2021). One study confirmed that ingestion of microplastics led to reduction of body length in daphnia magna (Jemec et al., 2016). Long term exposure to LDPE microplastics led to decrease in body length of *Artemia salina* (Kokalj et al., 2021).

Table 4.3: Body lengths on Artemia salina in control and in mono and coexposure

Experiments	**Body length (um)**
Control	700
HDPE	678
LDPE	612
PP	620
Coexposure	**508**

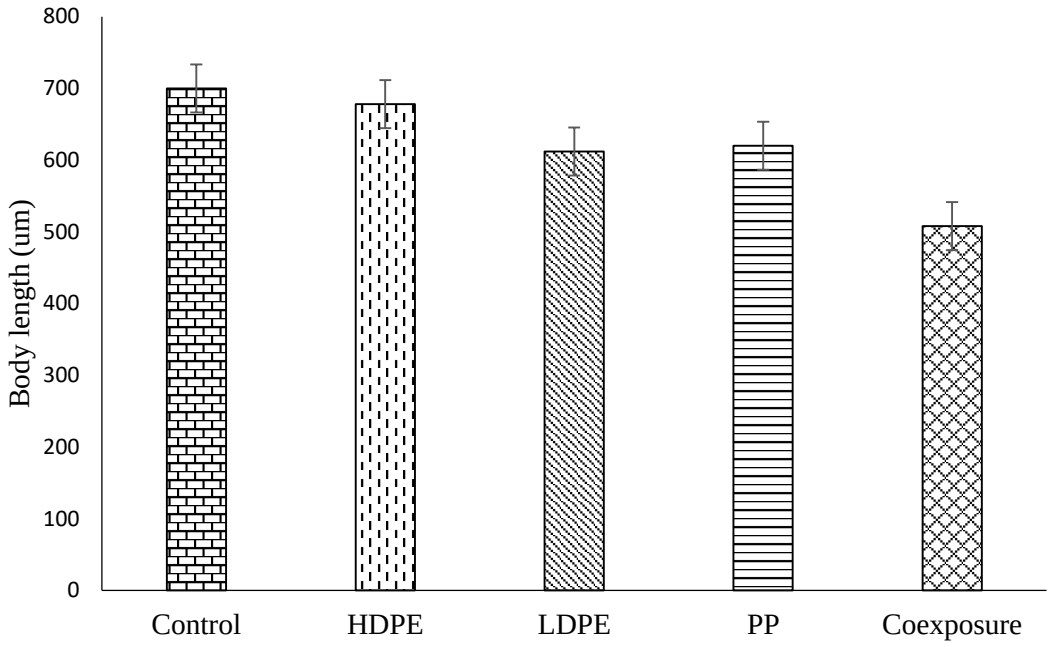


Figure 4.11: Body lengths on Artemia salina in control and in mono and coexposure

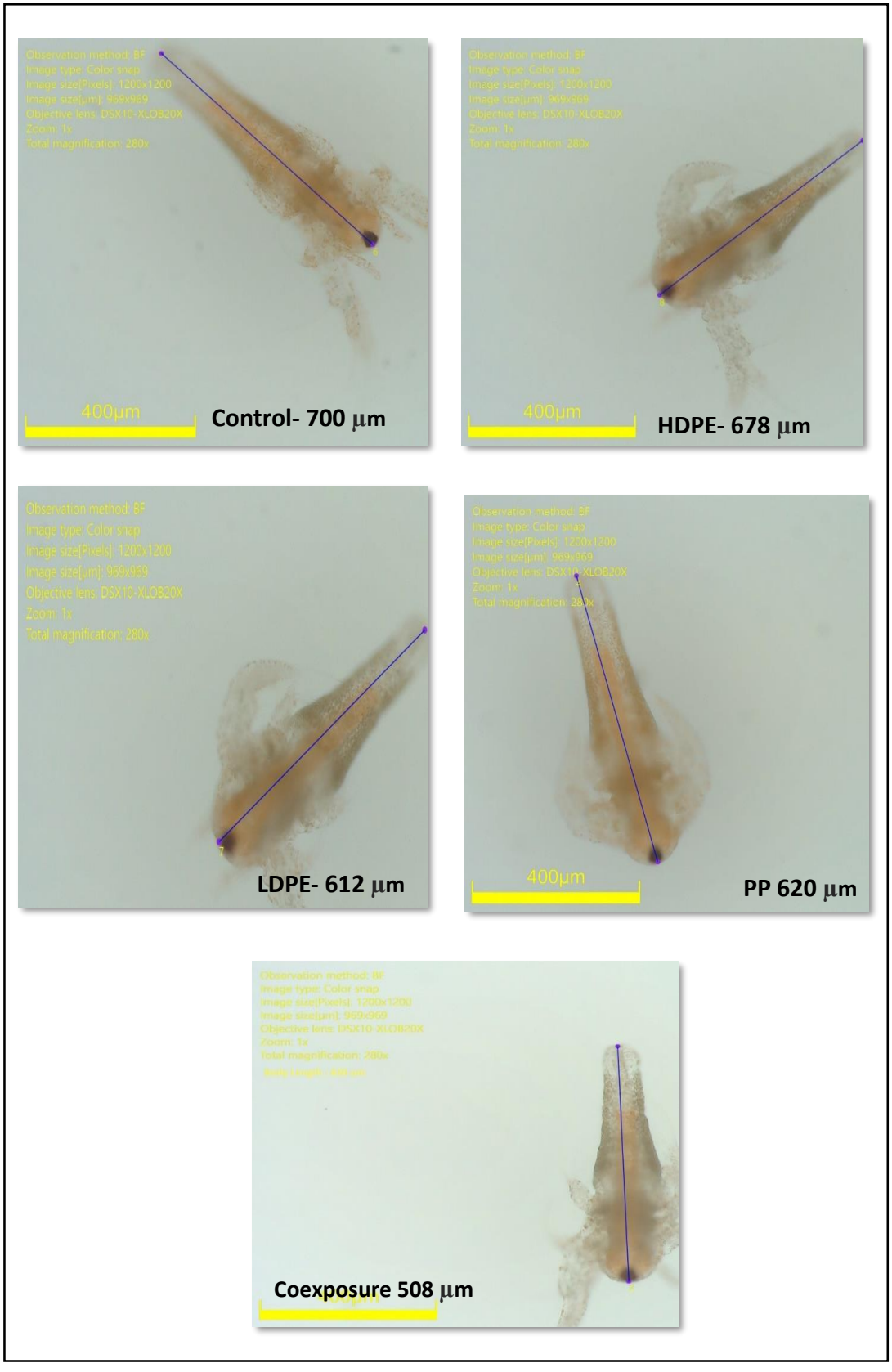


Figure 4.12: Body lengths of Artemia salina measured through DSX microscope

4.3.4. Impact of microplastics on swimming behavior

Swimming velocity of *Artemia salina* in mono exposure was inversely proportional to increasing concentrations. *Artemia salina* were moving freely in microplastic free water but they tend to stay at bottom in microplastic exposures. The swimming velocity was also very good when there were no microplastics present. But the movement decreased as the concentration of microplastics was increased and was decreased even more in coexposure experiment. Table 4.4 and 4.5 show graphs of swimming patterns of *Artemia salina* in mono and coexposure in low and high concentrations. According to Suwaki and coworkers (2020), the presence of polystyrene microspheres altered the swimming performance of the pelagic copepod T. turbinata at both low and high MP concentrations. Similar results were also reported previously by (Gambardella et al., 2017). These results were in line with the results of this study.

Table 4.4: Swimming patterns of Artemia salina in mono exposures (PP, LDPE, HDPE) and coexposure in low concentration

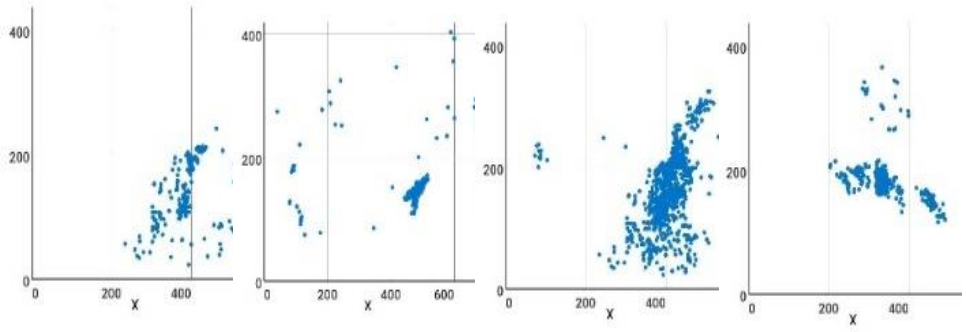
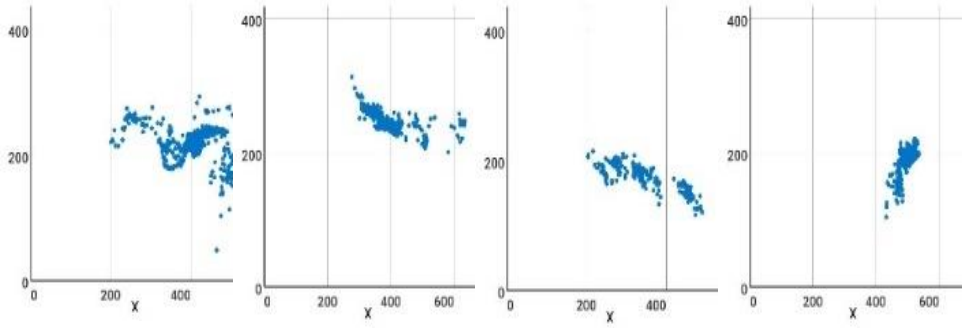
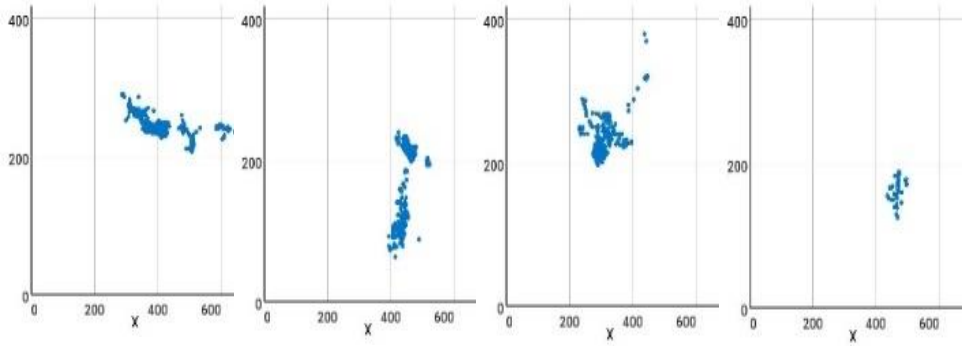
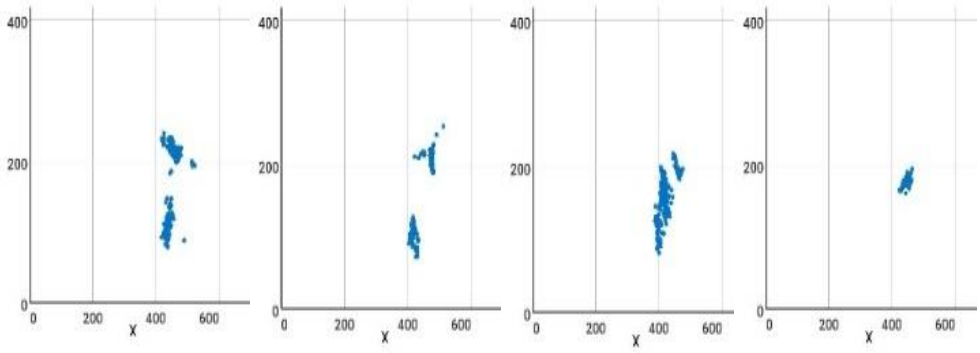
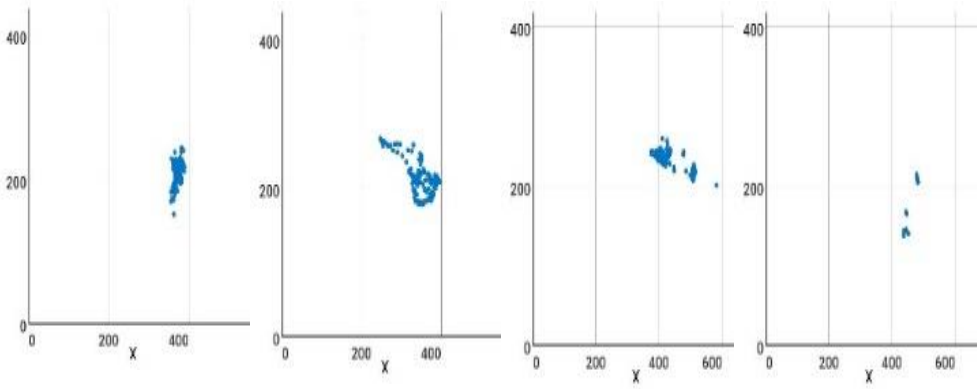
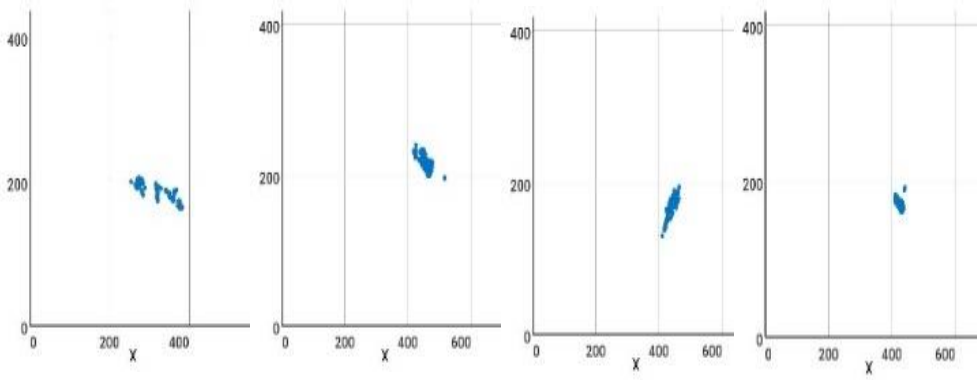
Concentration (mg/l)	LDPE	HDPE	PP	COEXPOSURE
0				
25				
50				

Table 4.5: Swimming patterns of Artemia salina in mono exposures (PP, LDPE, HDPE) and coexposure in high concentration

Concentration (mg/l)	LDPE	HDPE	PP	COEXPOSURE
75				
100				
125				

4.3.5. Bioaccumulation of microplastics in *Artemia salina*

The gut is the most important target for marine organisms, especially zooplankton. In this study, microplastics were ingested and eliminated by brine shrimps after exposure. Nile Red dyed microplastics were clearly seen under the fluorescent microscope that confirmed the ingestion of microplastics. The consumed microplastic were accumulated in the gut of brine shrimp during acute toxicity. The number of particles ingested per organism were almost same in lower concentrations in both mono and coexposure experiments when counted under microscope but the accumulated particles increased in higher concentrations with maximum value in coexposure. Similar results were shown by (Canniff & Hoang, 2018) in which daphnia magna ingested a smaller number of particles in lower concentrations.

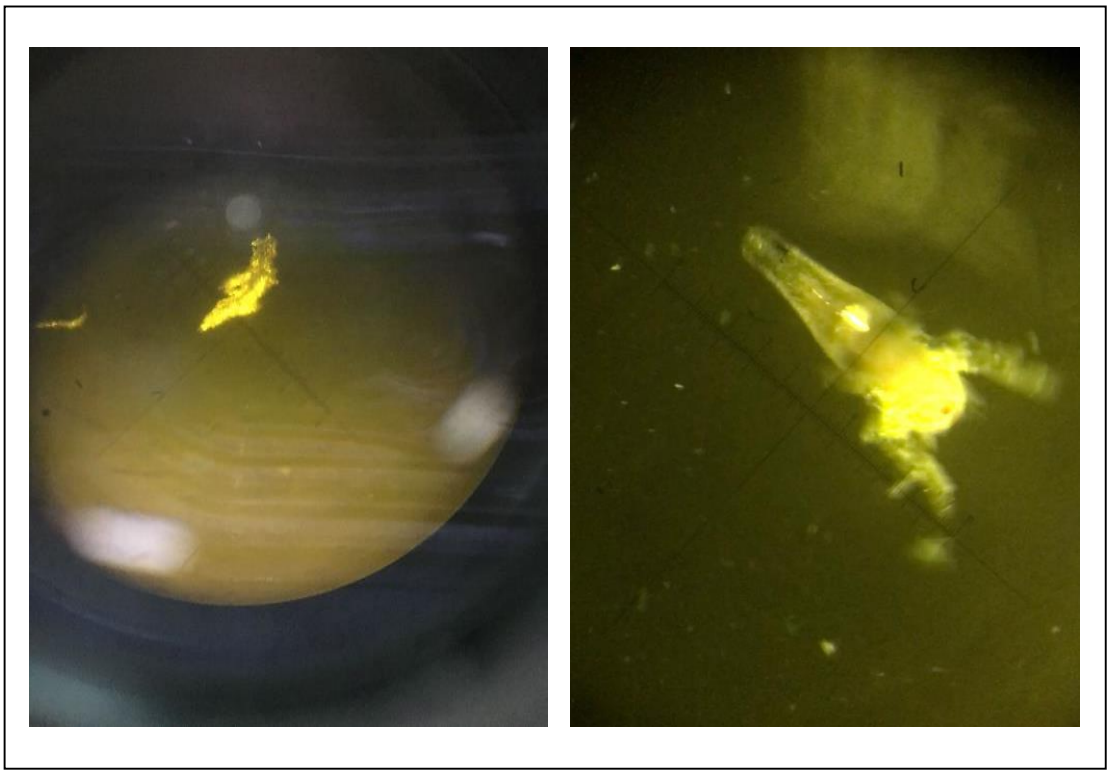
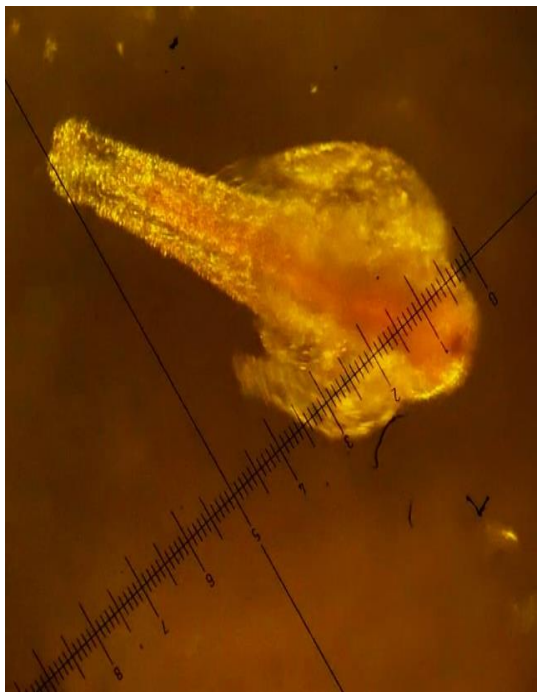
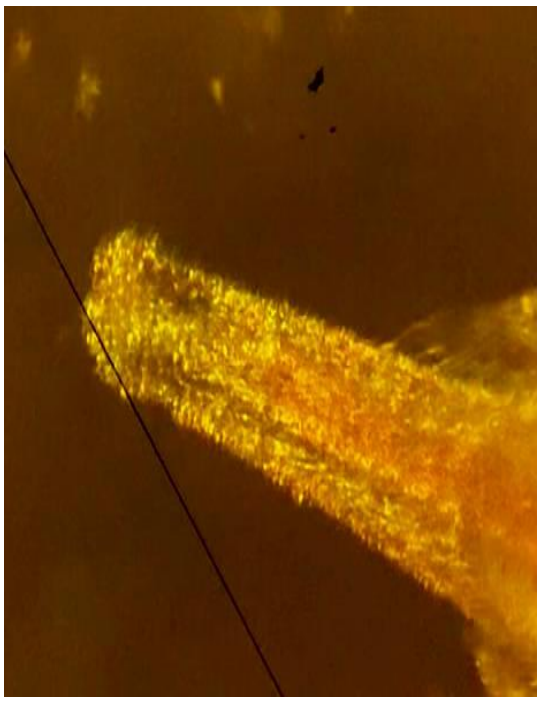


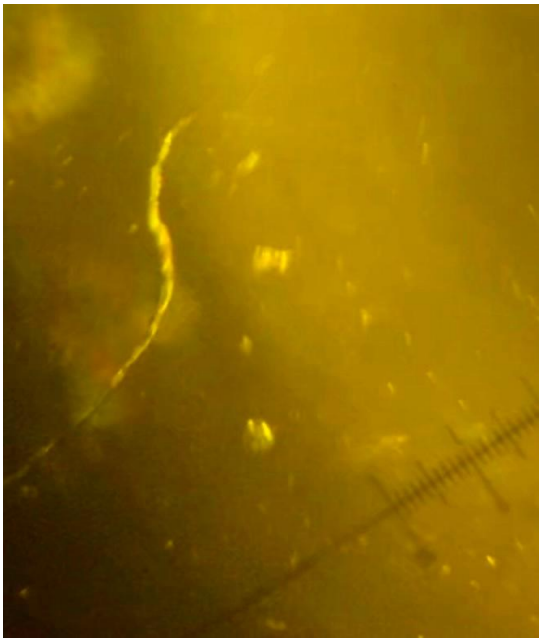
Figure 4.13: Picture of dyed microplastic and microplastic in gut of Artemia salina when viewed through florescent microscope under 4X lens



(a)



(b)



(c)

Figure 4.14: (a) Microplastics accumulated in Artemia salina body. (b) A close picture of Artemia salina gut filled with microplastics. (c) Microplastics excreted from Artemia salina body.

Table 4. 6: Number of particles of LDPE accumulated in Artemia salina

Concentration (mg/l)	Total particles ingested	LDPE particles bioaccumulated
0	0	0
25	7-12	0.95
50	11-16	1.35
75	15-22	1.85
100	23-29	2.6
125	28-35	3.15

Table 4. 7: Number of particles of HDPE accumulated in Artemia salina

Concentration (mg/l)	Total particles ingested	HDPE particles bioaccumulated
0	0	0
25	7-12	0.85
50	11-16	1.25
75	15-22	1.7
100	23-29	2.35
125	28-35	2.85

Table 4.8: Number of particles of PP accumulated in Artemia salina

Concentration (mg/l)	Total particles ingested	PP particles bioaccumulated
0	0	0
25	7-12	0.7
50	11-16	1.25
75	15-22	1.8
100	23-29	2.2
125	28-35	2.7

Table 4.9: Number of particles of microplastics accumulated in Artemia salina in coexposure.

Concentration (mg/l)	Total particles ingested	Particles bioaccumulated in coexposure
0	0	0
25	7-12	0.95
50	11-16	1.35
75	15-22	1.85
100	23-29	2.85
125	28-35	3.5

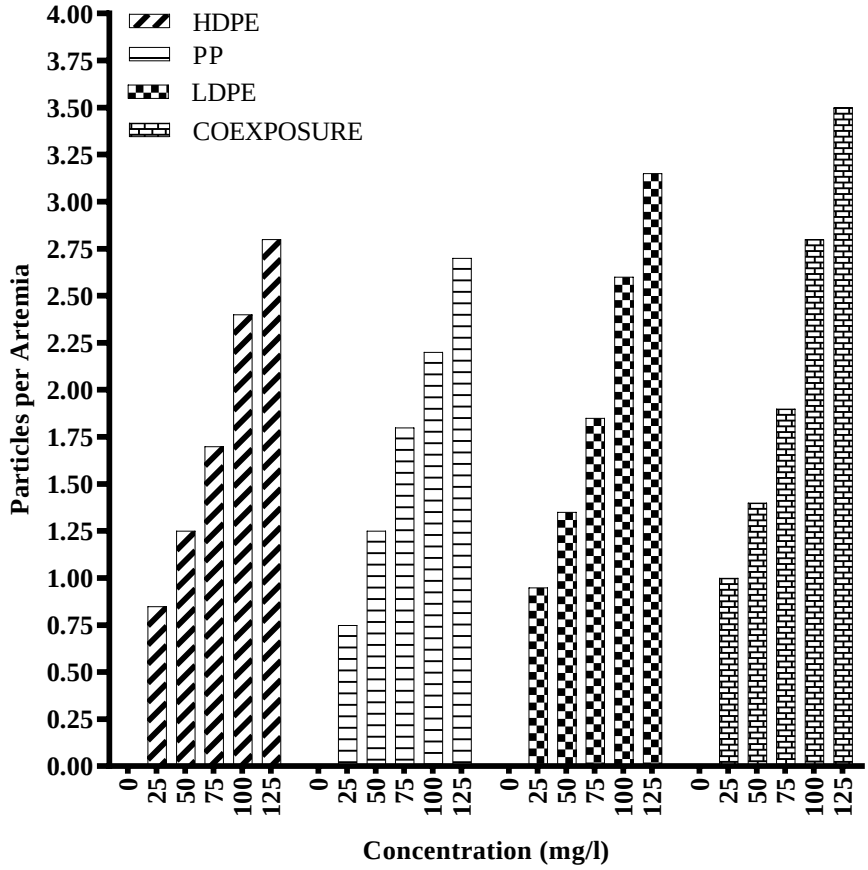


Figure 4.15: Number of particles of microplastics accumulated in Artemia salina in mono exposure & coexposure

The results clearly indicated that as the concentrations of microplastics increased, the number of particles per organism also increased, regardless of whether the exposure was individual (mono) or combined (coexposure) (figure 4.12). Particles per *Artemia salina* were almost the same in HDPE and PP exposures but higher in LDPE exposure. Overall coexposure values were highest with 3.5 particles per *Artemia salina* in 125 mg/l. Increased particles in *Artemia salina* over increasing concentrations over a period of 24 hours demonstrates that the accumulation of particles in *Artemia salina* have a concentration dependent relationship. This type of relationship was also studied on A. parthenogenetica over both short term (24 h) and long term (14d) exposure time (Wang et al., 2019). Moreover, such correlations have also been discovered in tadpoles (Xenopus tropicalis) and sea urchin larvae (Hu et al., 2016; Kaposi et al., 2014). Although they are nonselective filter-feeders and ingest anything that is of right size

but the results of Raman Spectroscopy revealed that in the presence of all three microplastics PP, LDPE, HDPE i.e. coexposure experiment *Artemia salina* ingested PP more in lower concentrations followed by LDPE and HDPE during their motion in water whereas, in higher concentrations they ingested HDPE more which suggests that the combined presence of different microplastics may lead to synergistic effects *on Artemia salina*'s feeding behavior, enhancing or inhibiting the ingestion of certain microplastics.

Table 4. 10: Results of Raman spectroscopy

Concentration (mg/l)	polymers ingested by *Artemia salina* in coexposure
25	PP> LDPE> HDPE
50	PP> LDPE> HDPE
75	PP> LDPE> HDPE
100	HDPE>PP>LDPE
125	HDPE>PP>LDPE

5. CONCLUSIONS & RECOMMENDATIONS

Conclusions

The global issue of marine microplastics pollution has raised significant concerns among researchers and environmentalists. Extensive studies have been conducted to assess the impact of microplastics on aquatic organisms, particularly marine crustaceans such as *Artemia salina*. This study focused on the exposure of *Artemia salina* nauplii to various types of microplastics, both individually and in combination. The results of this research highlighted a disturbing trend: when these microplastics were present together, their detrimental effects on *Artemia salina* were magnified, surpassing the harm observed when each type of microplastics was examined.

The findings showed that when these microplastics were co-exposed, their toxic effects on *Artemia salina* were amplified, surpassing the toxicity of each type when tested individually. This co-exposure led to a more stressful environment for the crustaceans, resulting in decreased survival rates, higher mortality rates, and increased accumulation of microplastics within their bodies. Additionally, the presence of all microplastics significantly reduced the swimming movements of *Artemia salina*. Overall, the ingestion of microplastics by marine primary consumers poses severe consequences, considering their crucial role as a vital food source in the marine ecosystem. To address this pressing issue and ensure the survival of various aquatic organisms, including *Artemia salina*, the establishment of global networks for managing plastic waste in coastal and marine habitats is of utmost importance.

Recommendations

1. The underlying mechanisms of how PP, LDPE, and HDPE interact with *Artemia salina* at the molecular and cellular levels should be investigated. This could involve transcriptomic, proteomic, or metabolomic analyses to elucidate specific pathways affected.

2. Comprehensive toxicological assessments should be conducted including studies on oxidative stress, genotoxicity, and endocrine disruption, to understand the full range of potential impacts on *Artemia salina.*
3. The effects of microplastic aging and weathering on the toxicity of PP, LDPE, and HDPE to *Artemia salina* can be studied as aging can alter their chemical composition and surface properties.
4. The combined effects of these plastic types with other environmental stressors commonly found in aquatic ecosystems, such as pollutants, temperature variations, or pH changes should be explored to better mimic real-world conditions.

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