Isolation and Identification of Microplastics and Nanoplastics in *Gallus Gallus Domesticus* and *Paphia Malabarica*

A Dissertation Report for

Course code and Course Title: GBO-381Dissertation

Credits: 8

Submitted in partial fulfilment of the requirements for the degree of

Master of Science in Biotechnology

by

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I hereby declare that the data presented in this dissertation report entitled, "Isolation and Identification of Microplastics and Nanoplastics in *Gallus gallus domesticus* and *Paphia malabarica*" is based on the results of investigations carried out by me in the Biotechnology Discipline at the School of Biological Sciences and Biotechnology, Goa University under the Supervision of Dr. Sanjeev C. Ghadi and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will be not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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This is to certify that the dissertation report "Isolation and Identification of Microplastics and Nanoplastics in *Gallus gallus domesticus* and *Paphia malabarica*" is a bonafide work carried out by Ms. Shreesiddhi Vinod Bhomkar under my supervision in partial fulfilment of the requirements for the award of the degree of Master of Science in Biotechnology in the Discipline of Biotechnology at the School of Biological Sciences and Biotechnology, Goa University.

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ACKNOWLEDGMENT

Foremost, I would like to express my sincere gratitude to my guide, Dr. Sanjeev C. Ghadi (Prof. School of Biological Sciences and Biotechnology) for his guidance and immense knowledge.

I would also like thank my my professors, Dr. Savita Kerkar, Dr. Meghanath Prabhu, Dr. Samantha Fernandes, Ms. Dviti Mapari and Ms. Snesha Bhomkar for their encouragement and support. I express my deepest gratitude to Dr. Dharmendra Tiwari for his constant guidance on using epifluorescence microscope.

I am wholeheartedly grateful to Ms. Veda Manerikar for her advice and insightful comments throughout the dissertation. I also am grateful to all the other Ph.D. scholars for their help and suggestions.

Also, I would like to extend my sincere esteems to all our non-teaching staff Mr. Serrao, Mr. Ashish Kurtiker, Mr. Samir Malik, Ms. Sandhya, and Ms. Jaya for providing help in some or the other way.

I am thankful to Birla Institute of Technology and Science for providing me data on Raman spectra.

A special thanks to my friends and fellow colleagues for all the support and encouragement.

Lastly, I would like to heartily thank my parents, Mr. Vinod Bhomkar and Mrs. Manpreet Bhomkar, and my sibling, Ms. Samruddhi Bhomkar for their constant love and encouragement.

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LIST OF ABBREVIATIONS

µm	Micrometer
mm	Millimeter
mL	Milliliter
nm	Nanometer
℃	Degree(s)Celsius
%	Percentage
<	Less than
MP	Microplastic
NP	Nanoplastic
NR	Nile Red
PP	Polypropylene
PU	Polyurethane
PS	Polystyrene
PA	Polyamide
PVC	Polyvinylchloride
PET	Polyethylene terephthalate
KOH	Potassium hydroxide
FTIR	Fourier transform infrared spectroscopy
PE- HD	Polyethylene, high-density
PE-LD	Polyethylene, low-density
PAA	p-(acrylic acid)
pEVA	poly (ethylene-vinylacetate)

CONTENTS

CHAPTER 1:

INTRODUCTION &

LITERATUREREVIEW

1.1 Introduction

Plastics are extremely versatile products and are widely used in a wide range of applications due to their low density, low thermal/electrical conductivity, and resistance to corrosion. (Frias & Nash, 2019) However, these properties also allow them to persist in the environment for tens to hundreds of years. In 2019, around 368 million metric tons (MMT) of plastics were manufactured worldwide, with half of them produced in Asia. These numbers are expected to continue to increase every year. (Tiseo, 2021)

In the life cycle of plastics, they undergo degradation through various processes such as mechanical, chemical and biological degradation, and break down into smaller fragments called microplastics. Microplastics have become a global concern due to their widespread distribution in various ecosystems, including aquatic and terrestrial environments. (Lusher, 2015) Ingestion of microplastics by organisms can potentially impact their feeding, digestion, excretion, and reproduction processes. (Habib, et al., 2022) Furthermore, during the manufacturing process, additive chemicals such as flame retardants, pigments, antimicrobial agents, heat stabilizers, UV stabilizers etc. are added to the plastic products. As plastics progressively degrade, the surface area to volume ratio increases leaching the additive chemicals into the environment posing harm to the ecosystems. (Teuten, et al., 2009) In addition, microplastics provide a stable habitat for different harmful microorganisms and pathogens for a long period of time, increasing the bioavailability of pathogens to lower trophic species such as bivalves. (Zhang, et al., 2022)

Microplastics are categorized into primary and secondary types. Primary microplastics are originally produced to be <5 mm in size, while secondary microplastics result from the breakdown of larger items.(Katare, et al., 2022). Recently, it was proposed the size category of MP 1~1000 μm. (Hartmann, et al., 2019). They are further classified based on types of polymers like polypropylene (PP), polyvinyl chloride (PVC), polystyrene (PS), polyurethane (PUR), and polyethylene terephthalate (PET) and polyethylene (PE) (high and low density). Based on the type, microplastics can be further divided into fragments, pellets, fiber, plastic film, foamed plastic and Styrofoam. (Hidalgo-Ruz, Gutow, Thompson, & Thiel, 2012) Moreover, depending on the hydrophobicity, plastics can be divided into non-polar (LDPE, HDPE, PP, PS) and polar (nylon, PVC PET) (Gao, Wontor,

& Cizdziel, 2022). Further, nanoplastics are derived from degradation of single microplastic particles. Nanoplastics size range from 1 to 1000nm, due to which they can pass through biological membranes easily making them a complicated issue around the world. (Hernandez, Yousefi, & Tufenkji, 2017) Nanoplastics have been overlooked due to their lack of its definition and quantification. Nevertheless, it has been suggested that nanoplastics could be the most hazardous due to their high potential for bioaccumulation and biomagnification. It has been proved that if the plastic matter is small enough to cross the nuclear membrane surrounding the DNA, it can cause mutagenic processes that are considered to play a role in the carcinogenesis of cells. (Rubio, Marcos, & Hernández, 2020).

The various sources of plastics are milk and juice jugs (PE-HD); plastic bags, six pack rings, bottles, netting, and drinking straws (PE-LD); rope, bottle caps, and netting (PP); plastic utensils, food containers (PS); nylon fabric (PA); film, containers and pipes (PVC); plastic beverage bottles (PET) etc. (Smith, Love, Rochman, & Neff, 2018)

Over the last decade, microplastics have emerged as novel pollutants that have a negative impact on food security. Microplastics can be transported across different environmental compartments and trophic levels of the food web, finally entering the human body. Ingestion of contaminated food is the chief pathway through which microplastics are exposed to humans. Microplastics have been found in a range of food consumed by humans, including chicken (Leon, et al., 2022); fish, mussels, oysters, shrimps and other crustaceans (Gabriel, Vethaakd, Lavorantea, & Lundebyef, 2018); milk products (Filho, et al., 2021), and drinking water. (Schymanski, Goldbeck, Humpf, & Fürst, 2018) Among seafood products, the consumption of shellfish such as clams, oysters, and mussels, poses a higher risk of transfer to humans. (Guzmana, et al., 2022) The accumulation of microplastics in the body is known to affect the gastrointestinal system, impair epithelial permeability, cause localized inflammatory processes and change the composition of gut microbiota. (Campanale, Massarelli, Savino, Locaputo, & Uricchio, 2020) However, only those microplastics with the appropriate shape and size will be ingested by organisms and the impact of the microplastics will depend on exposure concentration, particle type and retention time. (Lu, et al., 2016)

Broiler chickens *(Gallus gallus domesticus)* are an important source of protein around the world. They are bred to have uniform size specifically for meat yield. Chickens have a unique structure of the digestive system that includes crop, gizzard and glandular stomach which makes the digestion process of microplastics after being ingested by avian species is completely different from that of fish and mammals. (Kai, et al., 2022). Microplastics found in free-range chicken is likely a result of ingestion of earthworms and other organisms soil polluted with microplastics. (Lwanga, et al., 2017) Microplastics can accumulate in chicken gut through chicken feeds. Microplastic contamination has been observed in feed pellets made of mixture of byproducts like seafood and crop. (Cabansag, Olimberio, & Villanobos, 2021). Other possible exposure routes through which plastic particles enter the bodies of the chicken are ingestion of water and respiration of air. (Mourik, Veen, Velzen, Groenewoud, & Leslie, 2022) Research on microplastics and nanoplastics in chicken gut is still lacking.

Shellfish are a very popular seafood and are widely distributed in coastal areas. They are valuable sentinel organisms for indicating levels of different pollutants in the environment therefore the measurement of microplastics in clams could be a direct way to evaluate the environmental risks caused by microplastic uptake. (Su, Cai, Kolandhsamy, Wu, & Rochman, 2018) Since they are typical benthic filter feeders. (Wang, Li, & Wang, 2021) they have the ability to concentrate and accumulate pollutants substantially above background environmental levels. This is because filter feeders tend to act as a trap, accumulating pollutants because of their low excretion rates. (Jara-Marini, et al., 2013) *Paphia malabarica,* also known as short neck clam, is found in marine and estuarine habitats. It is an economically important species of clams in India, and is used for human consumption especially in Kerala. The uptake of microplastics in shellfish is known to occur during filter feeding (Li, Ma, Zhang, & Shi, 2021) These particles are often found accumulated in their digestive tracts and as they are consumed whole, it is more likely to expose microplastics to the human diet. (Smith, Love, Rochman, & Neff, 2018)

In Goa, two major rivers, Zuari and Mandovi form an estuarine system with their interconnecting canal (Qasim & Gupta, 1981). The Zuari estuary represents a very rich coastal ecosystem for fishery resources supports a large number of economically important

species. This is because the margins of Zuari estuary have dense mangrove vegetation filled with silt, clay and detritus that has been carried by riverine influx. *Paphia malabarica* is collected throughout the year in the estuary by hook and line fishing and skin diving. (Singh, Sreekanth, & Manju, 2015)

A standard protocol for identification and analysis of microplastics still does not exist. The most commonly used techniques based on literature are fluorescence microscopy, μFTIR spectroscopy and Raman spectroscopy. (Xu, Thomas, Luo, & Gowen, 2019) Fluorescence microscopy is widely used for identification of microplastics because it allows easy detection and immediate visualization of the particles. The most common dye used for staining is Nile Red, which has gained widespread use due to its inexpensive nature and its affinity to a wide range of polymers. (Prata, et al., 2021) It is a lipophilic and solvatochromic dye whose emission spectrum depends on the polarity of the stained medium. Nile Red stains polar plastic polymers (e.g., nylon, PET, PVC) present fluorescence in the red range once stained, whereas hydrophobic polymers (e.g., PE, PP, PS) exhibit fluorescence in the yellow range once stained. (Prata, et al., 2021) Nile Red adsorbs on the surfaces of the polymers and renders them fluorescence when excited at certain wavelengths. The adsorption is generally due to hydrophobicity, electrostatics, van der Waals force, hydrogen bonding, and other factors between dyes and the polymer. (Aoki, 2022)

FTIR and Raman spectroscopy are analytical methods that confirm the composition of the microplastics and provide no false positive or negative data. Moreover, small plastic particles (less of 20μm) can be detected with μ-FTIR and μRaman spectroscopy. However, these methods are expensive and time consuming. (Mariano, Tacconi, Fidaleo, Rossi, & Dini, 2021)

There has been extensive research on microplastics in seafood but the study of microplastics in food sources obtained from terrestrial and estuarine ecosystems is still at an early stage. For accurate assessment of the health risks associated with the consumption of microplastic-contaminated food, current information gaps must be filled. Therefore, in this study, microplastics in gut of broiler chicken and short necked clams from Zuari estuary were investigated.

1.2 Literature Review

Consumption of poultry and seafood meat contaminated with plastics is the most likely pathway for concentration of microplastics and nanoplastics in humans. The assessment of microplastics and nanoplastics in chicken and shellfish is important to understand their impacts on human health (Yee, et al., 2021).

Avian is one of the main species endangered by microplastics, as they are usually apex predators and are more likely to accumulate MPs in their bodies. This was proved in a study of the common kingfishers along the Ticino River in northern Italy which detected MPs in their pellets (Winkler, Nessi, Antonioli, & Laus, 2020) Kai et al. recently showed exposure to PS microplastics disrupted the digestive system in chickens, resulting in intestinal tissue damage and intestinal flora disturbance. Since the digestion process of microplastics after being ingested by avian species is completely different from that of fish and mammals due to their unique digestive system, the results of the effects of MPs on fish and mammals cannot correctly infer the response of birds (Kai, et al., 2022).

Emerging research has shown that microplastics can accumulate in the gastrointestinal tracts of birds, including poultry. Research on microplastics in the chicken gut is still limited, but preliminary studies have shown evidence of their presence. However, there is no available literature on the nanoplastics found in guts of chicken. Reports from few countries have revealed that microparticles in terrestrial ecosystems have contaminated domestic chickens including crops and gizzards that are consumed as food (Lwanga E. H., et al., 2017). The occurrence of microplastics has been reported in a few studies in the gastrointestinal tracts of chickens using various analytical techniques such as microscopy, spectroscopy, and chemical analysis (Huanga, Chapmanb, & Denga, 2020). Moreover, it was found that under laboratory conditions, plastics ingestion by chickens reduced food consumption and the volume of the gizzards since plastic particles are well retained in the gizzards (Ryan, 1988). Based on the literature, different types of microplastics, including fragments, fibers, and films, have been found in the chicken gut (Leon, et al., 2022). Water and feed used in chicken production systems act as potential pathways for microplastics to enter the chicken gut (Habib, et al., 2022). A study found detectable concentrations of plastic particles in feed pellets (Mourik, Veen, Velzen, Groenewoud, & Leslie, 2022).

Research on microplastic pollution and the precise characterization of microplastics in estuarine ecosystems has begun only recently and is at an early stage. Although, several studies have been conducted on bivalves, *M. edulis* and *M. galloprovincialis* are the most studied bivalve species regarding MP contamination in recent years. Research on the abundance of microplastics from other commercially important bivalve species such as *Meretrix meretrix, Ruditapes philippinarum, C. gigas and Patinopecten yessoensis* have also been carried out. However, not much research is done on *Paphia malabarica* (Li, Ma, Zhang, & Shi, 2021).

One of the studies on Asian clam (*Corbicula fluminea*), in Taihu Lake, China showed variation in microplastic concentrations among different sampling sites within the same estuary. The results of this study suggest that the Asian clam can serve as a bioindicator of microplastic pollution in freshwater systems (Su, Cai, Kolandhsamy, Wu, & Rochman, 2018).

Research shows that fibers are the most commonly occurring shape of microplastics in a majority of shellfish species, followed by spherical particles. (Wang, Li, & Wang, 2021) In addition, the main polymer compositions of MPs present in these shellfish species are PE, PP, PS & PET. (Li, Ma, Zhang, & Shi, 2021) The sources of the microplastics found in shellfish could be microbeads in personal care and cosmetic products, fibers from ropes and fishing nets, domestic sewage, and foam from household appliances and parts and disposable packaging boxes. (Helm, 2017) However, the data on the occurrence of nanoplastics in seafood is completely lacking.

A case study in Goa examined abundance of microplastics in the water, shellfish and finfish of Sal estuary, in which *P. malbarica* was examined for the first time. Low amounts of microplastics were found in clams as compared to other filter feeders such as oysters and mussels. It was inferred more the volume of water filtered, the more microplastics could be accumulated. (Sahaa, et al., 2021)

The methods described for identification and quantification of microplastics in literature include the following steps: (i) extraction and degradation of biogenic matter; (ii) detection and enumeration; and (iii) characterisation of the microplastic. Research shows that KOH could be adapted as the standard digestion technique for biological tissue digestions to

extract microplastics. (Thielea, Hudsona, & Russell, 2019) In a study conducted with six potentially suitable fluids for digestion of organic matter while retaining ingested plastics, potassium hydroxide (KOH) solution proved to be the most effective. (Dehaut, et al., 2016) Another study exposed 1M KOH solution to test its effects on different polymer types and origins, in the tissues of a small fish species. It confirmed that the different polymers remained unaffected from exposure to 1M KOH solution, which also held true for particles showing environmental degradation. However, substantial loss of mass was observed on low density polyethylene and cellulose acetate. (Kühn, et al., 2016) In other studies, 10% KOH was proved to be suitable for recovery of acrylic and rayon fibers, PP and PET in fiber-form and film of PVC and LDPE. Furthermore, it can be applied to dissolve various biological tissues, even when the particles are of single-micrometer size. It is also the most economical and least time-consuming method for dissolving tissues. (Thielea, Hudsona, & Russell, 2019) However, in sample having high fat content such as chicken, a soft gel like layer is formed on top of the suspension. This is because the lipids can undergo saponification in the presence of an aqueous alkali salt as KOH (Konkol & Rasmussen, 2015). A simple and inexpensive method was proposed in a study to optimize potassium hydroxide digestion which allows the whole viscera to be digested. In this study, the gel was treated with different dilutions of EtOH: KOH (1:1, 1:2, 1:4, and 1:10). 1:10 dilution showed most efficiency and great flexibility. Moreover, the addition of EtOH had no evident effects on the polymers (Dawson, Motti, & Kroon, 2020).

For quantification of microplastics, magnifying lenses or microscope visual sorting are some of the most broadly used techniques, especially for larger fragments (0.3 to 5 mm) (Maes, Jessop, Wellner, Haupt, & Mayes, 2017) In addition, direct smear can be applied to estimate the number of microscopic particles in a solution. The known volume of suspension with the particles to be counted, is spread over a known area. After the smear is dried, fixed and stained, the number of particles is carefully counted. This method is advantageous for counting microscopic particles that are either too small to be seen clearly in a chamber, needs no special apparatus and yet provides a fair degree of accuracy. Nonetheless, uneven distribution of the particles and tediousness of the counting can alter the quantification results. (Wang S. H., 1941)

There is limited literature addressing the quantification of nanoplastics in environmental samples. When nanoscale level of contamination is present in complex environment, they become extremely difficult to be detected. In a study, a possible solution to isolation and detection of nanoplastics was proposed. It used bioindicators as an approach to count and identify NPs on-a-chip. *C. robusta* individuals were exposed to different nanoplastics in varying concentrations dispersed in seawater, for a time period adequate to filter the entire sample volume. The results suggested that *C. robusta* had the ability to internalize and concentrate polymeric particles lesser than 1µm without affecting their size and shape. This method enabled the NP particles counting and identification by enzymatic digestion. The use of bioindicators was revealed to be a suitable methodology to monitor the nanoplastic pollution in marine environment due to their capacity to take up and accumulate nano-particles. (Valsesia, et al., 2021) According to a study, size-based differentiation for nanoplastics could be achieved by ultrafiltration or other separation methods such as flow field fractionation (FFF) and hydrodynamic chromatography (HDC). (Wallace, Alexandar, Bignami, & Cortrill, 2016)

In previous studies, the extracted microplastics were characterized according to colour, length and shape (fragment, fibre, bead and film). (De Witte B & Cooreman, 2014) Presently, the final assessment of the microplastics is achieved via µFourier transform infrared (FTIR) spectroscopy, µRaman spectroscopy and Pyrolysis-gas chromatography with mass spectrometry. However, these methods require expensive instrumentation, very long analysis time and may interfere with pigments. (Mariano S. , Tacconi, Fidaleo, Rossi, & Dini, 2021). Analytical methods for assessment of nanoplastics are yet to developed.

Fluorescence microscopy is also used to study microplastics, particularly for white and transparent MPs due to their innate ability to emit fluorescence. Several studies on microplastics using fluorescence microscopy have been done the last few years. For example, the ingestion of microplastics and nanoplastics by *T. japonicas* copepod was examined by fluorescence microscopy. (Lee, Murphy, & Hur, 2020) The literature lacks a standardized method for staining MPs to view under fluorescence microscope. A variety of dyes have been explored for microplastic staining, of which Nile Red is the most studied and widely used. A study tested multiple dyes (Oil red EGN, Eosin B, Rose Bengal,

Hostasol Yellow 3G and Nile Red) for their ability to adsorb to plastics. Nile Red was found to be the most effective in terms of adsorption and fluorescence intensity. (Maes, Jessop, Wellner, Haupt, & Mayes, 2017) Accordingly, it was proposed as alternative methods for quicker and more economical analysis of MPs in biological samples. (Cole, et al., 2013) It has emerged as a novel technique used for identification of MPs that label plastic particles, in particular when analyzing tissues. (Mariano S. , Tacconi, Fidaleo, Rossi, & Dini, 2021)

Nile Red (9-diethylamino-5H-benzo[α]phenoxazine-5-one) is lipophilic dye and was first used detecting intracellular lipid droplets. It was adapted for microplastic analysis by Andrady. (Andrady, 2010). Although this strategy does not reveal information on the chemical structure of the particles, it allows a quick to perform quantification of microplastics. Moreover, it is a relatively fast, simple, economical for the assessment of microplastic load in a sample. (Tamminga, Hengstmann, & Fischer, 2017)

Nile red staining can be used in various combinations, with each using different conditions such as concentrations, solvents, incubation times, and temperatures. One of the studies revealed that using higher concentrations of dye results in increased the fluorescence intensity of the dyed particles. Though, it also increased the background signal. The study found the optimum dye concentration to be 10µg/mL as it gave a good balance between visibility, speed and background signal; and the incubation time for visibility was determined to be between 30 to 60 minutes. (Maes, Jessop, Wellner, Haupt, & Mayes, 2017)

Nile Red is poorly soluble in water therefore organic solvents are generally used for preparation of Nile Red solutions. (Park, Oh, & and Hong, 2022) The hydrophobic properties of plastic allow them to exhibit fluorescence when excited with certain wavelengths. Likewise, Nile Red displays a pronounced sensitivity of its chromophore to alterations in solvent polarity. Nile Red exploits this property of plastics and can be used for detection of microplastics in environmental samples. (Kershaw, Turra, & Galgani, 2019) The maximum emission wavelength and the intensity of the fluorescence strongly depend on the solvent used for extraction. Hence, the color emitted by microplastics stained by Nile red varies depending on their surface hydrophobicity and can range from deep red

to strong yellow gold. (Gao, Wontor, & Cizdziel, 2022) Acetone, chloroform, dimethylsulfoxide, hexane, methanol, ethanol, and isopropyl alcohol are common solvents used to prepare the staining solutions. A recent study compared eight possible solvents-methanol, ethanol, ethyl acetate, acetonitrile, dichloromethane, toluene, n-Hexane, and cyclohexane for Nile Red to improve the staining approach for the quantification of microplastics. It was inferred that due to the relatively polar nature of Nile Red compared to plastics, the partitioning of its molecules from the carrier solvent to plastics can be more facilitated in non-polar carrier solvents such as n-hexane than in polar solvents. However, Nile Red was found to be less soluble in n-hexane than in acetone (Shim, Song, Hong, SH, & Jang, 2016) Therefore, Nile Red is more commonly diluted in acetone and is considered the optimal NR solvent for the development of the classification models in a study. (Rumin, et al., 2015) (Meyers, et al., 2022) Furthermore, a study evaluated and compared the benefits and efficiencies of acetone, chloroform and n-hexane as possible solvents for Nile Red. Chloroform was established to be the most suitable solvent in quantifying microplastics by accomplishing higher recovery rates. (Tamminga, Hengstmann, & Fischer, 2017)

Nile red-stained MPs can fluoresce when irradiated under UV, blue, green, and red incident lights with excitation wavelengths varying from 254 to 590 nm. In a study, three excitation and emission wavelengths, blue (excitation wavelength: 365 nm; emission wavelength: 445 nm), green-yellow (excitation wavelength.: 450–490; emission wavelength: 515–565 nm), and orange-red (excitation wavelength.: 534–558; emission wavelength: 590 nm) were investigated for identification of the Nile Red stained microplastics under a fluorescent microscope. This study exploited the solvatochromic nature of Nile Red, meaning that fluorescence emission spectrum shifts depending on the polarity of its environment. It was found that the maximum emission wavelengths of MPs stained with Nile red varied for different polymers. For example, the maximum emission wavelength for PE was 567 nm whereas for PP, PS, and PET were 571 nm, 593 nm, and 612 nm, respectively. Moreover, PP, PE, and PS exhibited yellow, orange, and red fluorescence respectively. It was observed that the peak emission fluorescence shifted from red to green with increasing hydrophobicity of the solvent and the type of microplastic. (Shim, Song, Hong, SH, & Jang, 2016) This was observed in an experiment wherein non-polar polymers

(LDPE, HDPE, PP,) stained by Nile Red showed strong green fluorescence while polar polymers (PE, PS, and PET) showed increased red fluorescence intensity with the increase in staining time. (Gao, Wontor, & Cizdziel, 2022) This indicates that the microplastics can be categorized based on their hydrophobicity. During analysis of stability of fluorescence, it was found that red fluorescence intensities of MPs stained by Nile red at room temperature reduce significantly after one month due to the easy desorption of dye from the surface of polymers. (Gao, Wontor, & Cizdziel, 2022) In addition, solutions such as KOH which are common in chemical digestions for microplastic extractions, have also been reported to weaken the fluorescence intensities of LDPE fragments, HDPE microbeads, and polyacrylonitrile fibers stained at 70◦C. (Karakolis, Nguyen, You, Rochman, & Sinton, 2019) According to literature, the wavelength of emission used may depend on the type of the polymer and the concentrations of the stain employed. For example, blue fluorescence is avoided for detection of certain polymers such as LDPE, PP, and EPS as they do not fluoresce in response to blue excitation. While Green-yellow fluorescence is suggested as the best choice for MP detection, it may also not be suitable for all polymers. (Gao, Wontor, & Cizdziel, 2022) Studies have found that PEST, PET, and PA stained by 5 µg/mL Nile red had a barely detectable, dim glow of green fluorescence and that polyurethane, PC, PVC, and PET dyed by 1 µg/mL Nile red exhibited weak green fluorescence. (Maes, Jessop, Wellner, Haupt, & Mayes, 2017)

It is important to note that biological tissues can also exhibit strong red fluorescence, which is one of the existing problems of using Nile Red staining for environmental samples. This could lead to overestimation of MPs present due to presence of residual organic matter. (Gao, Wontor, & Cizdziel, 2022)In addition, the presence of contamination from other materials such as biofilms (Cholewin´ska, et al., 2022) and grease can change the hydrophobicity of the MPs. (Gao, Wontor, & Cizdziel, 2022) Moreover, all solvents used for preparation of Nile Red tend to stain biogenic matter, which emphasizes the necessity to embed a pre-treatment for the destruction of biogenic matter into the operational protocol for microplastic identification. (Tamminga, Hengstmann, & Fischer, 2017) Therefore, it is necessary to eliminate these impurities as much as possible with adequate pre-treatment. These pre-treatments methods as discussed earlier efficiently remove surface impurities or contaminants and do not interfere with the chemical composition of the polymer. (Thielea,

Hudsona, & Russell, 2019) Nile red staining can thus be effective in differentiating MPs from non-plastic materials such as algae, seaweeds, wood, feathers, chalk and sand particles, mollusc shells, using only blue light microscopy filters (Shim, Song, Hong, SH, & Jang, 2016)

Rhodamine B (RhB) is another fluorescent dye that is extensively used in textile coloration. It has been used in research to fluorescently label particles of nano polystyrene, which can then be used to assess the potential toxicity of nano plastics on environmental organisms. (Qu, Xu, Li, Wong, & Wang, 2018) Research was done on RhB as a dye to identify its ability to stain plastics, and evaluate the efficiencies of distilled water, ethanol, and acetone, as possible solvents for dissolving RhB. Further, five different types of microplastics were labelled to investigate its applicability. The fluorescence stability of the dye in various conditions was also assessed. This study showed that ethanol was the best suitable solvent for RhB than distilled water and ethanol. It was also detected that RhB fluoresces strongly even at low concentrations. It stained particles of PE, PP, and PU were identified as amaranthine, and the stained PVC particles turned light pink. For PS, part of its outline was stained, but the colour changes in the other parts could not be easily visualized. Under a fluorescence microscope, at an excitation wavelength range of 450–490 and 515–565 nm, the particles showed green fluorescence with varying intensities. Additionally, RhB revealed to be fluorescently stable under varying conditions used in the experiment (light and gut fluid) and even in different solutions (KOH, nitric acid, and saturated NaCl). (Huiyan, Jiang, Zhong, & Hu, 2020)

In another study, the adsorption characteristics of RhB on various MPs were thoroughly investigated under various conditions of pH, salinity, humic acid and temperature. It was determined that PVC could adsorb more RhB than PS and PET as PVC was more polar than PS and PET. Moreover, the adsorption capacity of MPs increased with decreasing MPs sizes. (Du, Zhang, Jiang, & Wang, 2022) However, visual inspection of microplastics using fluorescent dyes may sometimes be inaccurate for smaller microplastics. Furthermore, for all visually identified items, it is necessary to confirm their plastic nature to avoid overestimations or underestimations of number of MPs in a particular sample.

This can be confirmed by μ-Raman spectroscopy and μ-Fourier transform infrared (FTIR) spectroscopy. (Hidalgo-Ruz, Gutow, Thompson, & Thiel, 2012)

Particle size an important factor in determining the extent and pathway of uptake of plastic particles in humans. There is a major knowledge gaps on effects of nanoplastics on environment and its exposure to humans. This is because very little literature is available on biological effects and interaction of nanoplastics at cellular level. Studies on presence of nanoplastics in food are yet to be performed.

CHAPTER 2:

AIMS AND OBJECTIVES

2.1 Aim

- To assess the presence of microplastics and nanoplastics in *Gallus gallus domesticus* and *Paphia malabarica*.

2.2 Objectives

- Isolation of microplastic and nanoplastics from gut of *Gallus gallus domesticus* (broiler chicken sample).
- Isolation of microplastic and nanoplastics from *Paphia malabarica* (a clams species from an estuary).
- Characterization and analysis of the microplastics and nanoplastics from the samples.
- Identification of the isolated microplastics polymer.

CHAPTER 3:

MATERIALS AND METHODS

3.1 Sampling sites

3.1.1 Chicken gut sample

Chicken gut sample was collected from a local broiler chicken retailer (15.2733976, 73.9475121) located at Mulgao, Salcete, South Goa.

3.1.2 Clams sample

Clams sample (*Paphia malabarica*) was collected from Zuari estuary, North Goa. (15.4078451, 73.9068629)

Fig 1. *Paphia malabarica*

3.2 Sample collection and preparation

3.2.1 Chicken gut sample

750g of chicken gut sample was collected from a local butcher shop in South Goa. The samples were then placed in a disinfected insulating box while being transported to the laboratory for analysis & testing. The sample was divided and transferred to two glass beakers. A 10% KOH (w/v) solution was added in the beaker containing chicken gut in the ratio of 3:1 (KOH: solution) and kept in the oven at 60℃ for 15 days till the organic matter digested completely.

To eliminate fat from the solution, it was stored at 20˚C for 24h. The solid fat layer formed at the top of the solution was separated. This was repeated until the fat layer stopped forming. To get rid of the remaining fat in the solution, it was treated with 100% EtOH.

Fig 2: Chicken gut digestion day 0

Fig 3: Chicken gut digestion day 8

Fig 4: Fat layer formed on the digested solution

Fig 5: Fat layer separated

Fig 6: Digested solution treated with 100% EtOH

3.2.2 Clams sample

Paphia malabarica were collected from the sampling site and placed in a disinfected insulating box while being transported to the laboratory for analysis & testing. Clams were subjected to ice for 60 minutes or till their shells were open. The soft tissue was excised from the shell using a scalpel, weighed, and transferred to a glass beaker for digestion. A 10% KOH (w/v) solution was added in the beaker in the ratio of 3:1 (KOH: solution) and kept in the oven at 60℃ for 2 days.

Fig 7: Clams digestion day 0

Fig 8: Clams digestion day 2

3.3 Isolation of microplastics and nanoplastics

3.3.1 Chicken sample

Once the chicken gut sample was completely digested, the solution was filtered using four sieves having pore sizes of 5 mm, 0.5mm, 0.2 mm and 0.044mm, kept above the other. The sieves were kept in hot air oven at 60˚C overnight. Microplastics were isolated from the sieves using forceps and placed in a clean petri dish. Magnifying glass was used to check the smaller microplastic particles, these were then collected using fine forceps. The isolated microplastics were washed with 100% ethanol, air-dried and observed under microscope.

For epifluorescence microscopic analysis of microplastics, 40µl of suspension was smeared over a slide (Wang S. H., 1941) . After air drying and fixation, the slides were stained using Nile Red for 30 minutes. The slide was observed under epifluorescence microscope using 10x objective and the number of particles was carefully counted. These slides were also observed under compound microscope.

For nanoplastic analysis, the filtered sample was further passed through 0.2µm sized Whatman nucleopore membrane using 20ml syringe. The membrane was kept for drying

overnight in a petridish. It was then stained using Rhodamine B and observed under epifluorescence microscope using 10x objective. Further, 40µl of suspension of the solution passed through the nucleopore membrane was smeared over a slide. After air drying and fixation, the slides were stained using Nile Red for 30 minutes. The slide was observed under epifluorescence microscope using 10x objective and observed for nanoplastics.

3.3.2 Clams sample

Once the sample was completely digested, the solution was filtered using four sieves having pore sizes of 5 mm, 0.5mm, 0.2 mm and 0.044mm, kept one top each other. The sieves were kept in hot air oven at 60˚C overnight. Microplastics were isolated from the sieves using forceps and placed in a clean petri dish. Magnifying glass was used to check the smaller microplastic particles, these were then collected using fine forceps. The isolated microplastics were washed with 100% ethanol, air-dried and observed under microscope.

For epifluorescence microscopic analysis of microplastics, 40µl of suspension was smeared over a slide (Wang S. H., 1941). After air drying and fixation, the slides were stained using Nile Red for 30 minutes. The slide was observed under epifluorescence microscope using 10x objective and the number of particles was carefully counted. These slides were also observed under compound microscope.

For nanoplastic analysis, the filtered sample was further passed through 0.2µm sized Whatman nucleopore membrane using 20ml syringe. The membrane was kept for drying overnight in a petridish. It was then stained using Rhodamine B and observed under epifluorescence microscope using 10x objective. Further, 40µl of suspension of the solution passed through the nucleopore membrane was smeared over a slide. After air drying and fixation, the slides were stained using Nile Red for 30 minutes. The slide was observed under epifluorescence microscope using 10x objective and observed for nanoplastics.

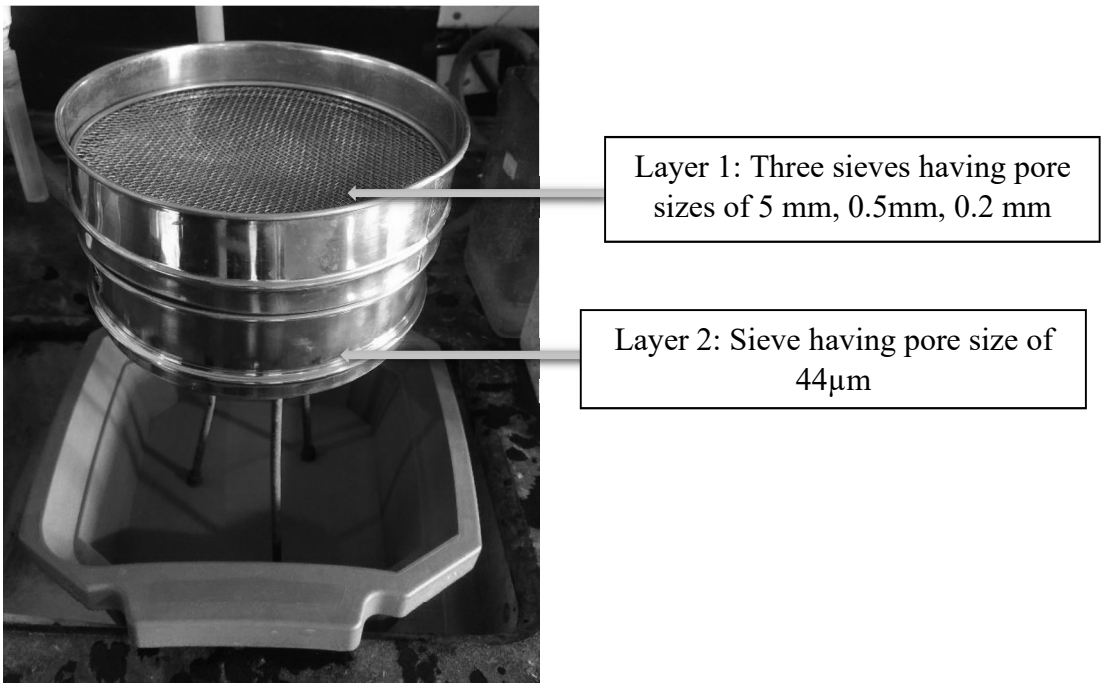


Fig 9: Double sieve set up for filtration of digested solutions

Fig 10: Sieves kept in hot air oven at 60˚C

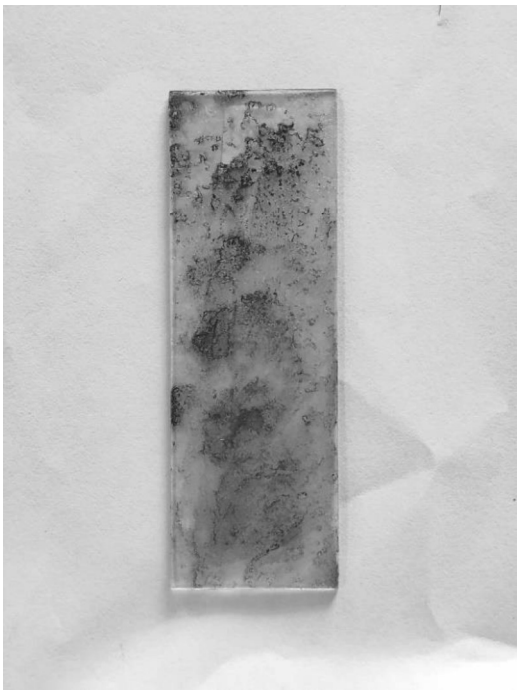


Fig 11: Slide stained with Nile Red

Fig 12: Filtered digested chicken gut solution passed through 0.22µm Whatman filter using a 20mL syringe

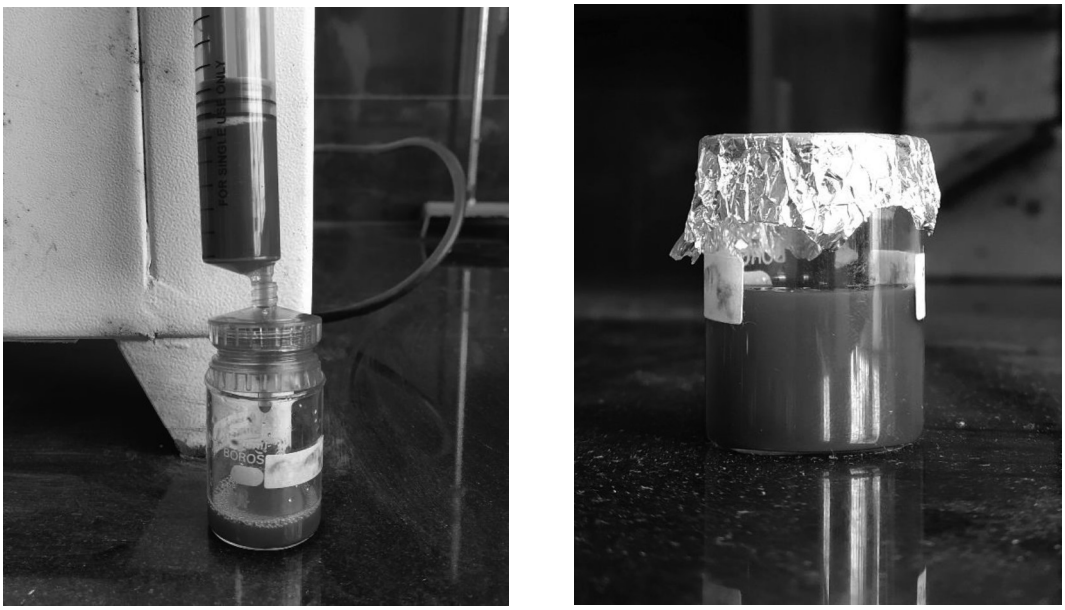


Fig 13: Filtered digested clams solution passed through 0.22µm Whatman filter using a 20mL syringe

3.4 Characterization of isolated microplastics

Microplastics were characterized based on texture, colour, hardness, shape and transparency.

3.5 Visualisation and analysis of obtained images

3.6.1 Compound microscopic analysis

Isolated microplastics were placed onto the slide, observed under 10X objective, and images were taken of the same.

3.5.2 Epifluorescence microscopic analysis

The slides and membranes stained with respective fluorescent dyes were observed under epifluorescence microscope in blue light (420-495nm) and examined for the color and intensity of the fluorescence the particles emitted. Microscopic scaling was done using ImageJ software using a hemocytometer as the reference.

3.5.3 Micro-Raman analysis

Each microplastic was given a code name based on the type of sample. Microplastics from chicken gut sample were given code names with the prefix CG which is denoted as Chicken Gut. Microplastics from clams sample were given code names with the prefix PM which is denoted as *Paphia Malabarica*. Similarity search for isolated particles was done using KnowItAll Information System 2021 by Wiley Online Raman Database.

CHAPTER 4:

RESULTS AND DISCUSSION

A] Chicken gut sample

After the digestion of 750g of chicken gut sample with 10% KOH, it was assessed for MPs of varying size ranges using microscopic and Raman spectroscopic techniques. In section 4.1, the MPs of size smaller than 4.9 mm and larger than 45µm were analysed, followed by analysis of MPs of size range between 44µm to 0.22µm in section 4.2. Lastly, detection of NPs of size less than 200nm was attempted.

4.1 Analysis of MPs (4.9mm to 44µm**)**

The microplastics were collected after passing through the sieves of pore sizes of 5 mm, 0.5mm, 0.2 mm and 44µm. The isolated MPs from the gut of chicken were visually categorized into groups based on their colour, level of thickness, transparency, hardness and shape, as shown in table 1. The particles were observed under compound microscope as shown in Fig.12-15. MPs from each category were further analysed by Raman spectroscopy as given in section 4.1.3.

4.1.1 Characterization of isolated microplastics

Table 1: Visual categorisation of isolated particles from chicken gut

Name of the Designated MP	Total number of MP particles	Color	Level of Thickness	Transparency	Hard/Soft	Shape of the MP
CG01	3	Brown	Thin	Transparent	Soft	Fragment
CG02	5	Colorless	Thin	Transparent	Soft	Fragment
CG03	8	Colorless	Thick	Transparent	Hard	Fragment
CG04	2	Brown	Thin	Opaque	Soft	Fragment

The total number of particles isolated after passing the digested chicken gut solution through the sieves were 18, of which all were in the shape of fragments. This is similar to the results found in the literature (Leon, et al., 2022). The colour of the isolated MPs ranged from colourless to brown.

.4.1.2 Microscopic analysis and scaling in light microscope

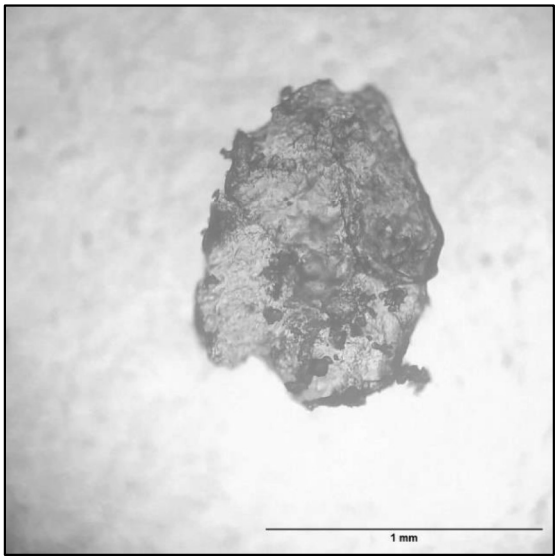


Fig 14: Microscopic image of CG01 MP

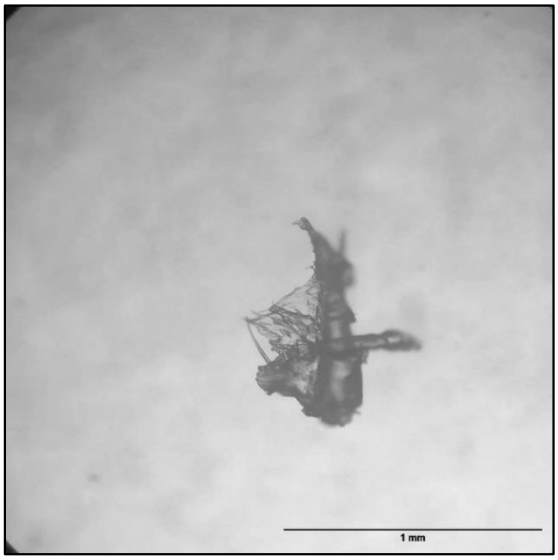


Fig 15: Microscopic image of CG02 MP

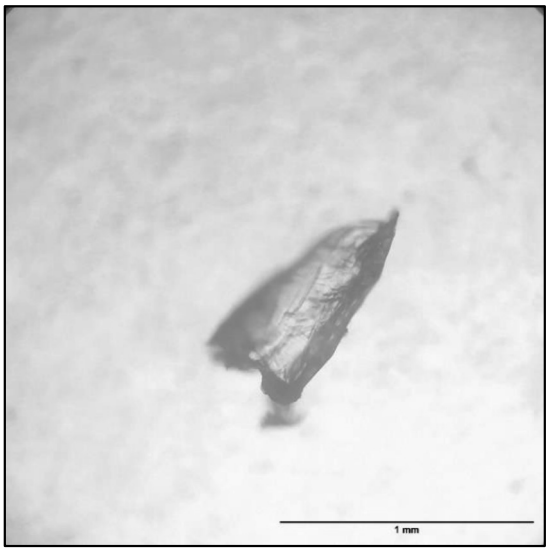


Fig 16: Microscopic image of CG03

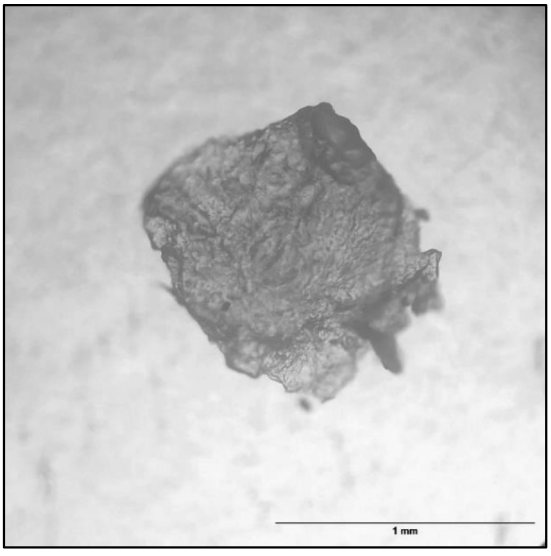


Fig 17: Microscopic image of CG04

As seen in fig 12-15, the MPs were observed under microscope using 10x objective were in the form of fragments and the sizes ranged from 0.5-1mm.

4.1.3 Micro-Raman Analysis

The similarity of Raman spectra of the isolated MPs of each category was compared to standard polymers using Wiley Science Solutions's KnowItAll Raman Spectral Database

Collection. The similarity varied from 75.3 to 91%. The results for each category are given in table 3.

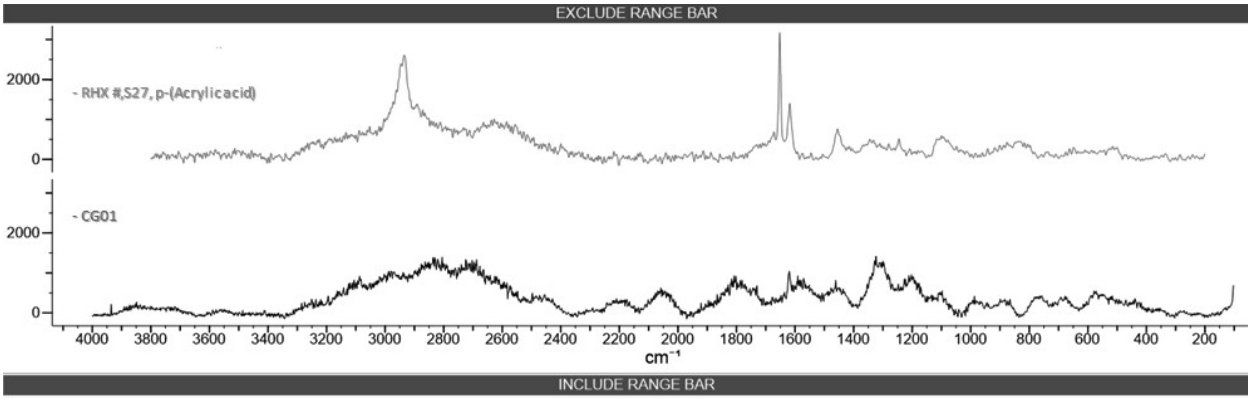


Fig 18: Raman spectrum for CG01

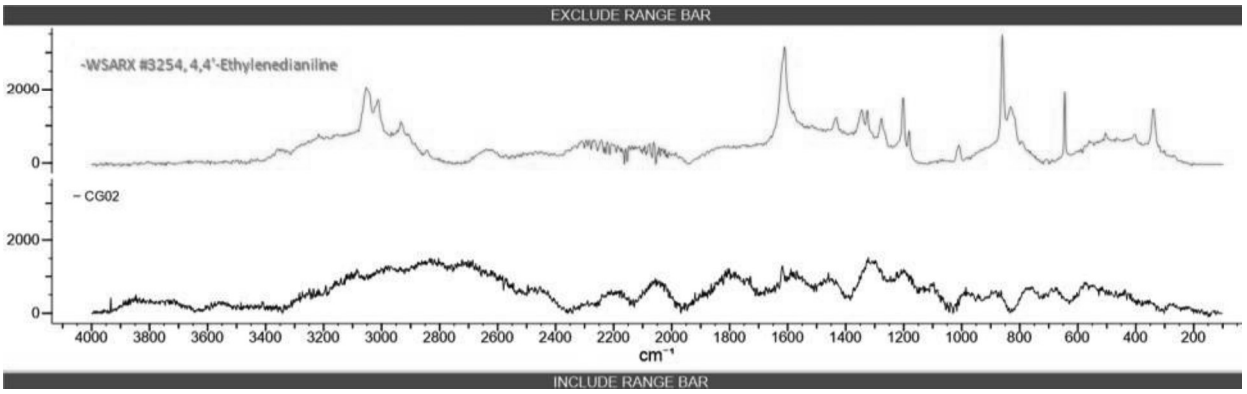


Fig 19: Raman spectra for CG02

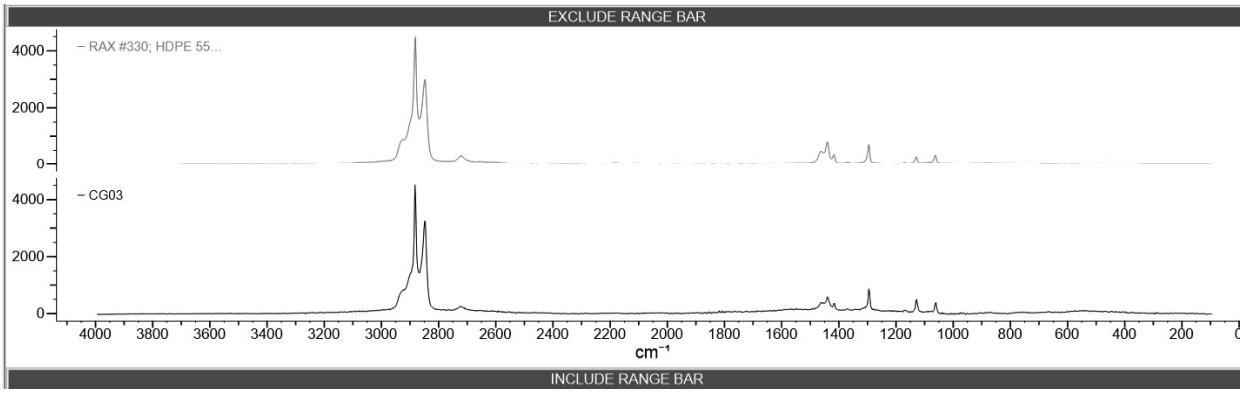


Fig 20: Raman spectra for CG03

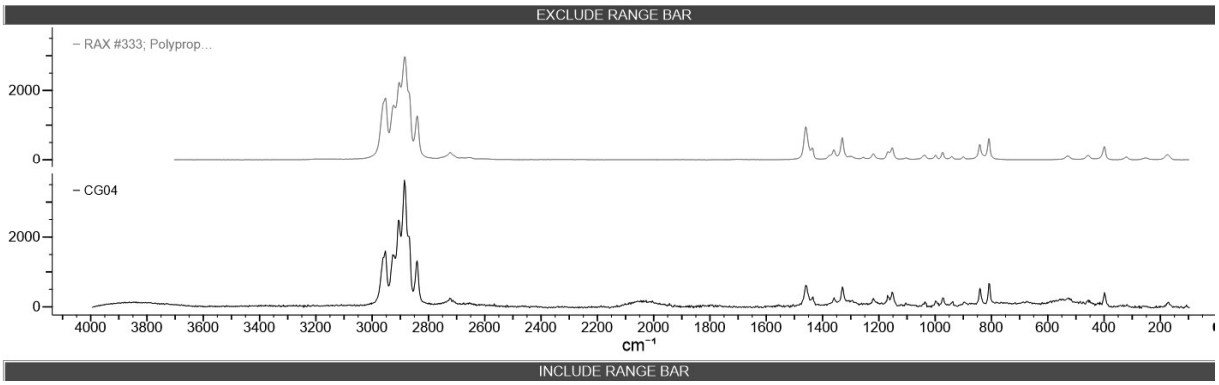


Fig 21: Raman spectra for CG04

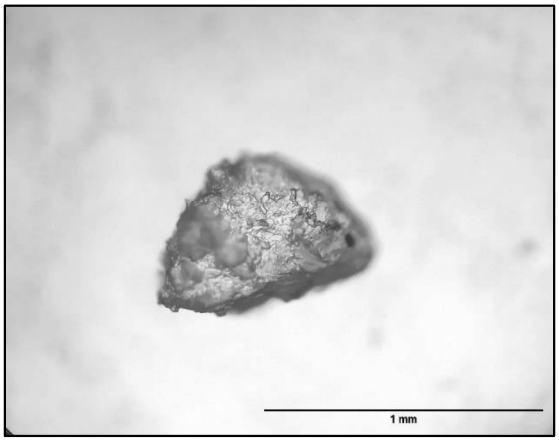
Table 2: µ-Raman analysis for chicken gut sample

Sr. no.	MP as given for µ-Raman	Similar to
1.	CG01	PAA
2.	CG02	4,4'-Ethylenedianiline
3.	CG03	HDPE
4.	CG04	PP

Raman spectrum for CG01 showed similarity to PAA and CG02 to 4,4'-Ethylenedianiline which is used as hardening agent for PU. CG03 was found similar to PP. CG04 was 90.48% similar to HDPE polymer. This is in accordance with results found in Leon, et al., (2022) in which HPDE MP particles were found in chicken meat.

4.2 Analysis of MPs (44µm to 0.22µm)

To verify the presence of microplastics, 40µl of suspension was smeared over each slide and air dried (Wang S. H., 1941). The slides were stained using Nile Red for 30 minutes and observed under light microscope using 10x objective, as shown in Fig 22A. The same slide was observed under epifluorescence microscope in which the background appeared dark and only particles emitted fluorescence as shown in Fig 22B, confirming the identity of the particle to be a microplastic. Thus, counting of MP particles of the sample was done using epifluorescence microscope. Based on the microplastic count obtained using direct smear method for 500µl of the sample, result was estimated for the remaining 495.5ml of the sample. The shapes and fluorescence exhibited by the particles are presented in Table 3 and depicted in Fig. 23 to 29.



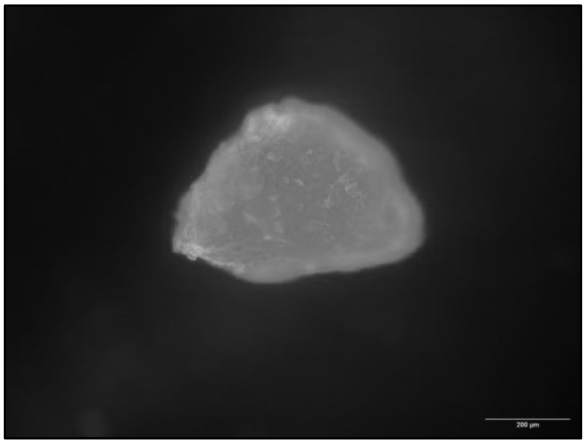


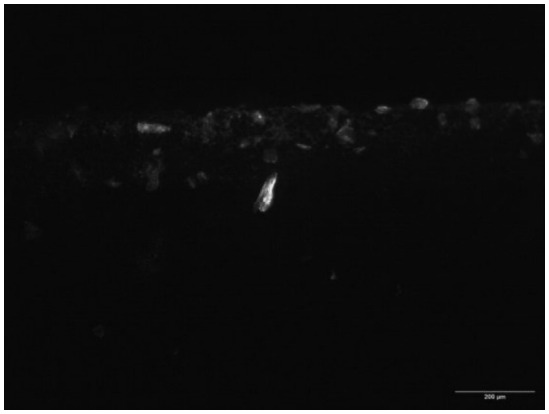
Fig 22A Fig 22B

Fig 22A: Microscopic image of MP in light microscope

Fig 22B: Microscopic image of MP in epifluorescence microscope

Table 3: Shapes and fluorescence emitted by particles in blue light (420-495nm)

Shapes of MPs particles	Fluorescence exhibited
Fibres, fragments, circular particles	Red, green, orange



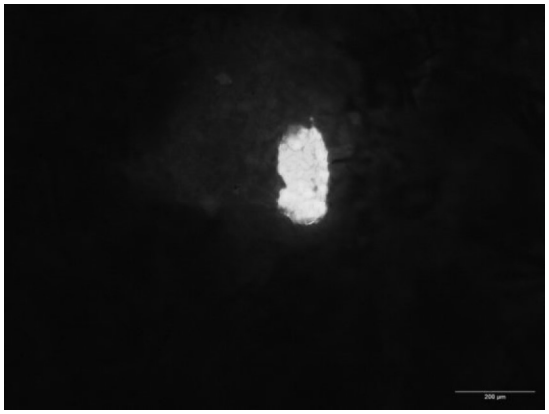
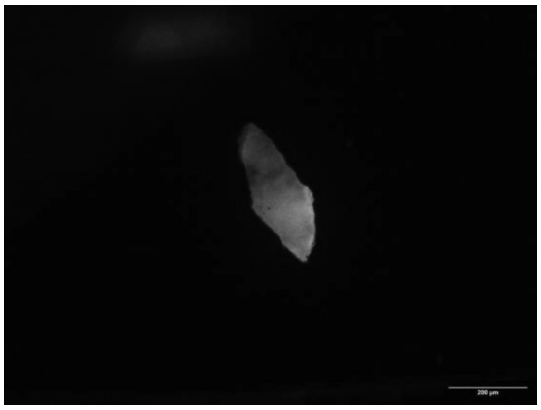


Fig 22 Fig 23



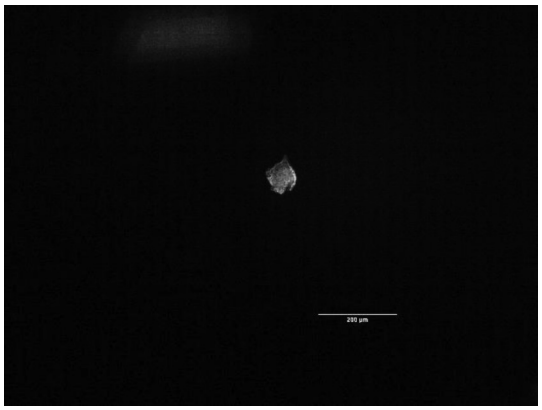
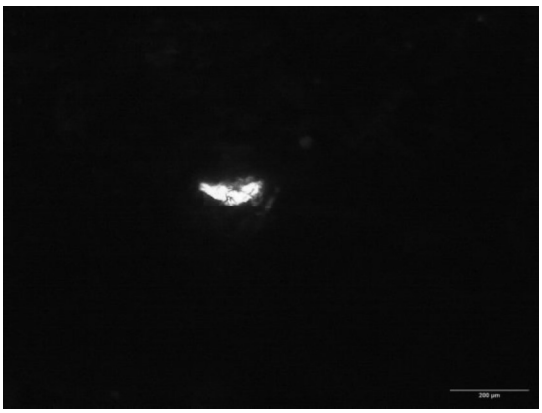


Fig 24 Fig 25

Fig 22-25: Microplastics showing green fluorescence under blue light



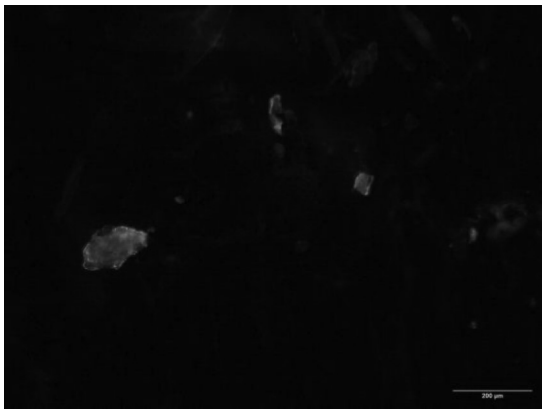
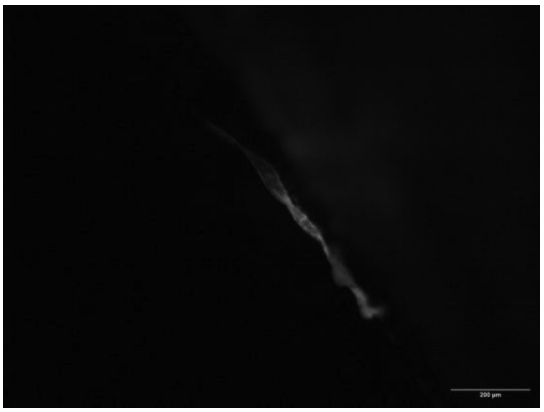


Fig 26 Fig 27



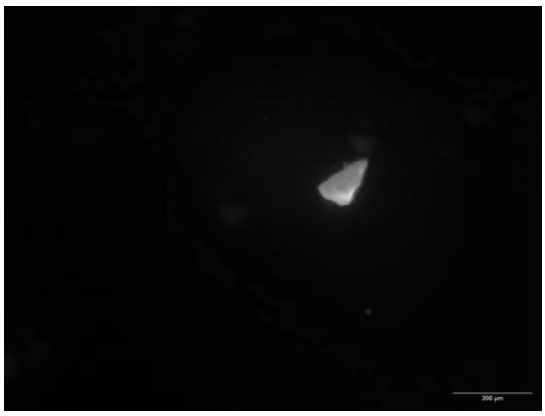
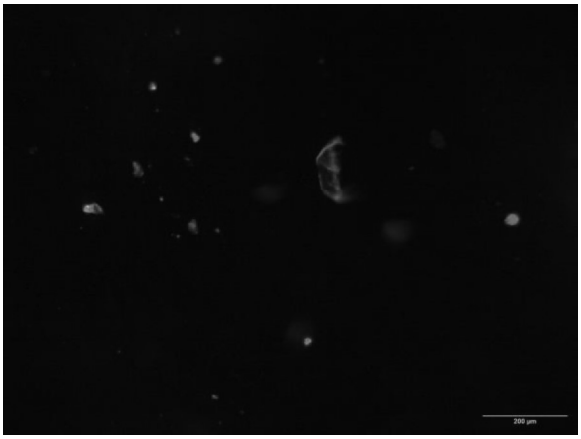


Fig 28 Fig 29

Fig 26-29: Microplastics showing red and orange fluorescence in blue light



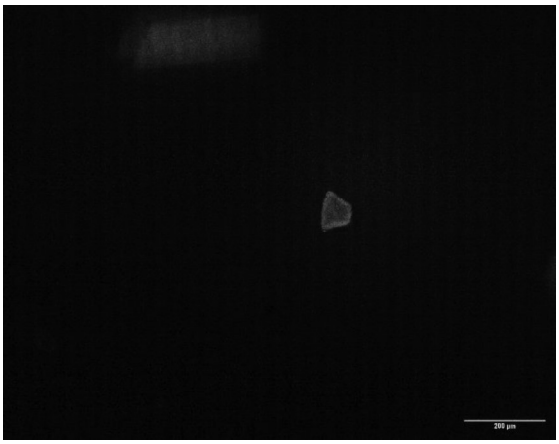


Fig 30 Fig 31

Fig 30-31: Microplastics on nucelopore membrane as stained with RhB

The total number of MP particles counted in chicken gut sample, under epifluorescence microscope was 486 in 500µl of the sample. Microplastics detected in the 500µl of the sample showed green, orange and red fluorescence in blue light under epifluorescence microscope when stained with Nile Red. The results from Raman spectroscopy showed the presence of both polar and non-polar polymers in chicken gut. This is in accordance with Gao, Wontor, & Cizdziel, (2022) in which and polar polymers and non-polar polymers were shown to emit red and green fluorescence respectively.

The filtrate was further passed through 0.22µm nucleopore Whatman membrane and left overnight for drying. It was then stained using Rhodamine B dye and observed in epifluorescence microscope using 10x objective. The observed fluorescence by particles with RhB was similar to that of Nile Red, as shown in Fig 30 and 31.

4.3 Nanoplastic analysis (<200nm)

40µl of filtrate passed through 0.22µm was smeared over each slide and air dried (Wang S. H., 1941). The slides were stained using Nile Red for 30 minutes and observed under light microscope using 10x objective for particles having <200nm size.

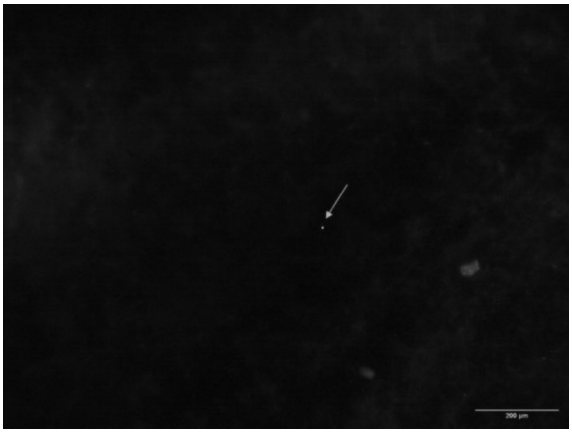


Fig 32

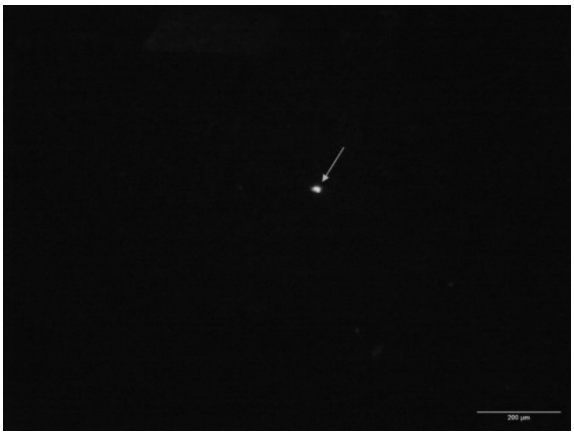


Fig 33

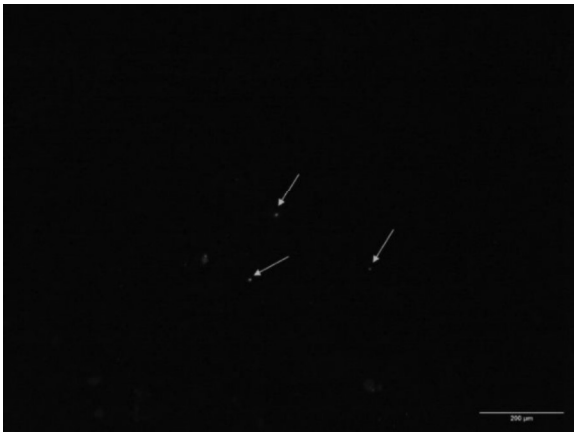


Fig 34

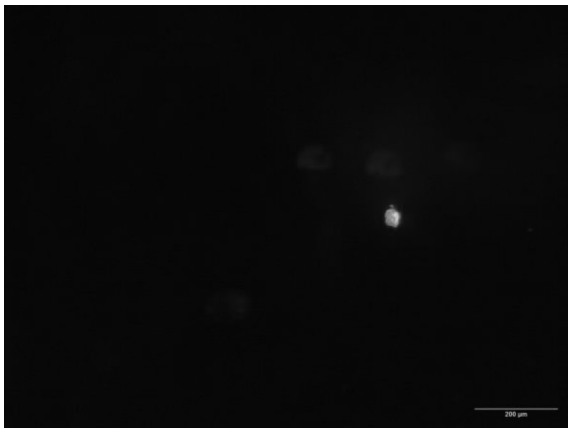


Fig 35

Fig 32-35: Nanoplastics showing different fluorescence in blue light

The smaller particles (< 200nm) emitted weak fluorescence ranging from red to green as seen in Fig. 32-35. However, in Fig 35 the particle size appears to be larger and the fluorescence intensity of the particle is higher. This could be because of aggregation of nanoparticles that looks like one single particle as described by Zhang, et al., (2022)

B] Clams sample

After the digestion of 62g of clams sample with 10% KOH, it was assessed for MPs of varying size ranges using microscopic and Raman spectroscopic techniques. In section 4.1, the MPs of size smaller than 4.9 mm and larger than 45µm were analysed, followed by

analysis of MPs of size range between 44µm to 0.22µm in section 4.2. Lastly, detection of NPs of size less than 200nm was attempted.

4.1 Analysis of MPs (4.9mm to 44µm)

The microplastics were collected after passing through the sieves of pore sizes of 5 mm, 0.5mm, 0.2 mm and 44µm. The isolated MPs from the clams were visually categorized into groups based on their colour, level of thickness, transparency, hardness and shape, as shown in table 4. The particles were observed under compound microscope as shown in Fig.36-39. MPs from each category were further analysed by Raman spectroscopy as given in section 4.1.3.

4.1.1 Characterization of isolated microplastics

Table 4: Visual categorization of isolated particles from clams

MP as given for µ-Raman	Total number of MP particles	Color	Thick/Thin	Transparent/Opaque	Hard/Soft	Similar to
PM01	2	Green	Thick	Opaque	Hard	Fragment
PM02	1	Brown	Thick	Opaque	Hard	Fragment
PM03	3	Colorless	Thick	Opaque	Hard	Fragment
PM04	6	Brown	Thick	Transparent	Hard	Fragment

The total number of particles isolated after passing the digested tissue of *Paphia malabarica* through the sieves were 12, of which all were in the shape of fragments. The colour observed of the isolated MPs were brown and green. The MPs of class PM03 were colourless.

4.1.2 Microscopic analysis and scaling in light microscope

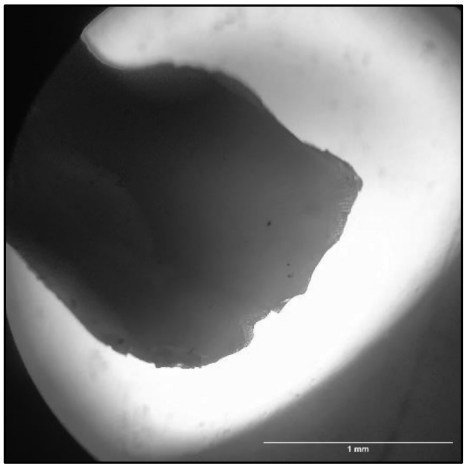


Fig 36: Microscopic image of PM01

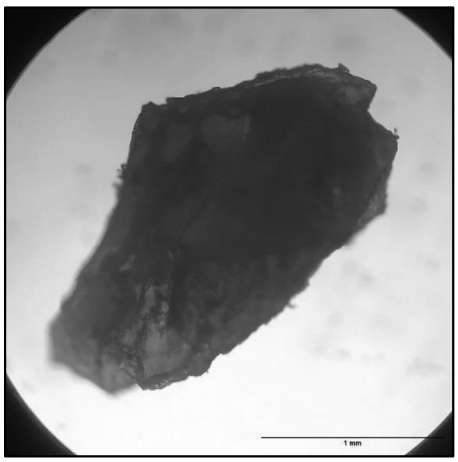


Fig 37: Microscopic image of PM02

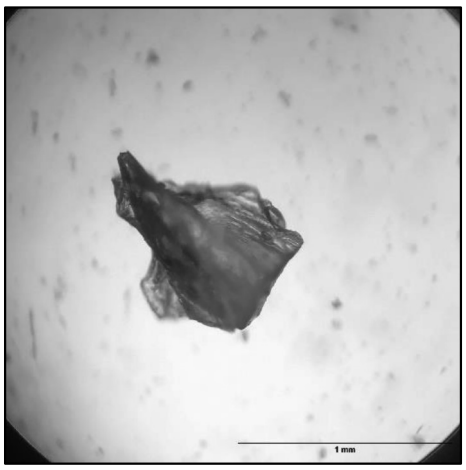


Fig 38: Microscopic image of PM03

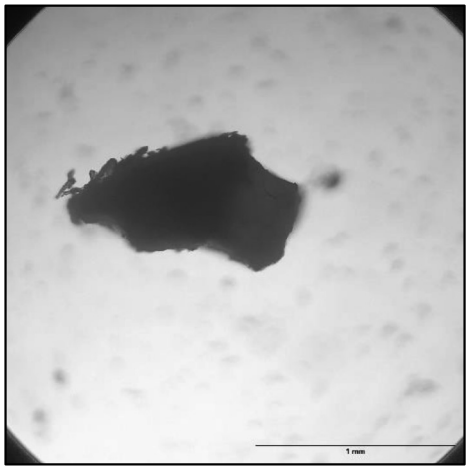


Fig 39: Microscopic image of PM01

As seen in Fig 36-39, the MPs were observed under microscope using 10x objective were in the form of fragments and the sizes ranged from 0.5-1mm.

4.1.3 Micro-Raman Analysis

Raman spectrum were compared to standard plastic polymer spectrum for each of the MP classes using Wiley Science Solutions's KnowItAll Raman Spectral Database Collection. The similarity varied from 75.3 to 91%. The results for each category are given in table 5.

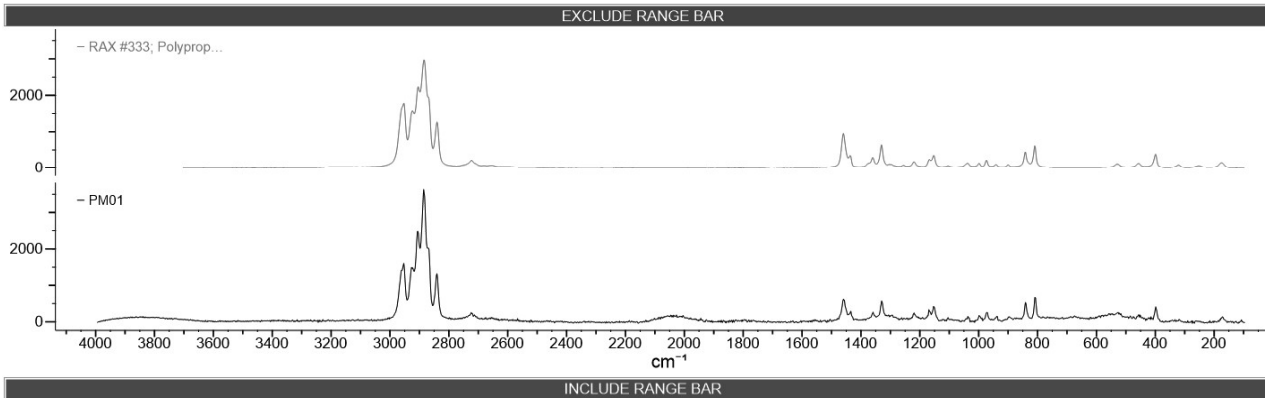


Fig 40: Raman spectrum for PM01

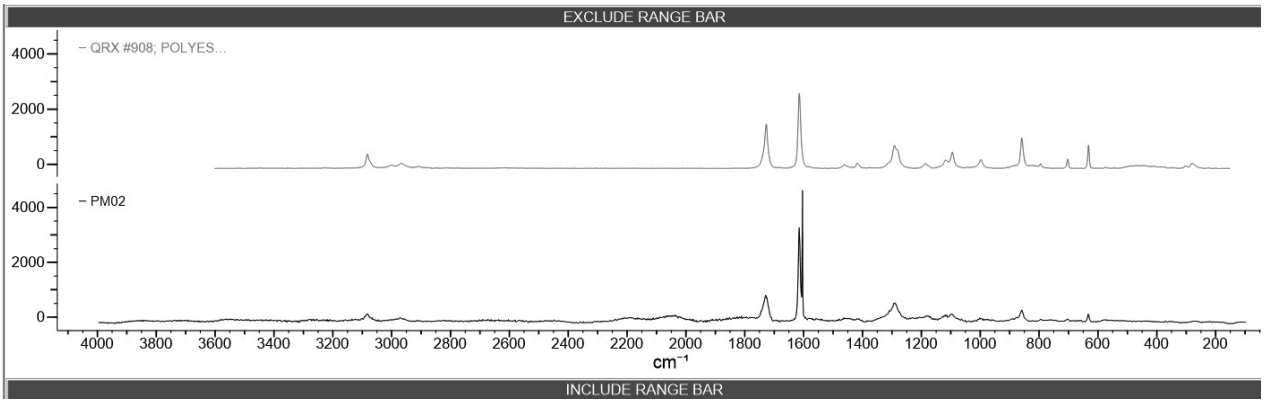


Fig 41: Raman spectrum for PM02

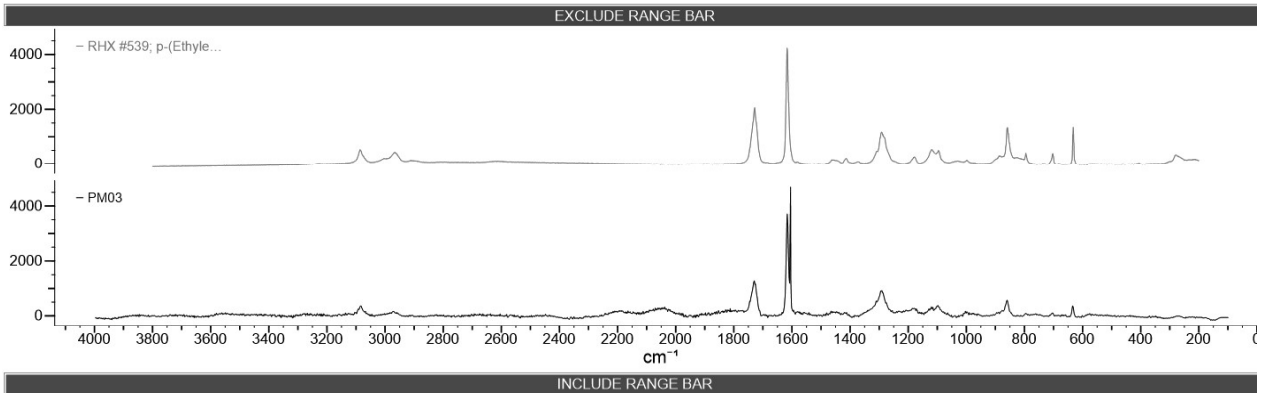


Fig 42: Raman spectrum for PM03

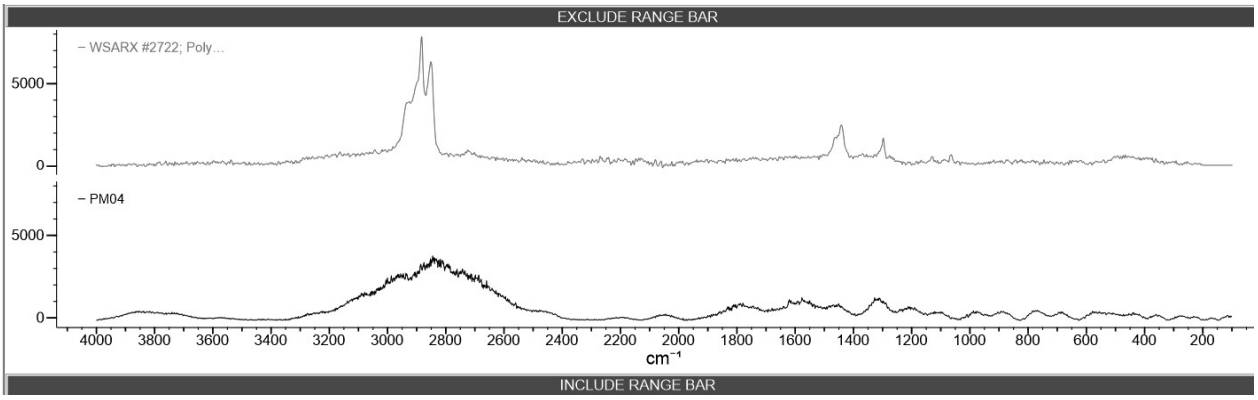


Fig 43: Raman spectrum for PM04

Table 5: µ-Raman analysis for clams sample

Sr. no.	MP as given for µ-Raman	Similar to
1.	PM01	Polypropylene
2.	PM02	Polyester
3.	PM03	PET
4.	PM04	Poly(ethylene-co-vinyl-acetate)

The similarity search of the spectra of the isolated MPs to polymers in Wiley's online database was in accordance with Li, Ma, Zhang, & Shi, (2021) where the main polymer compositions of MPs found in shellfish were PE, PP, PS & PET. The MPs in PM01 was identified to be similar to PP, PM02 showed similarity to polyester and PM03 was found similar to PET. This is in lines with the results obtained by Su, Cai, Kolandhsamy, Wu, & Rochman, (2018) wherein the most common chemical compositions in Asian clams from Yangzte river of China were polyester, followed by polypropylene and polyethylene. The MP in PM04 showed similarity to pEVA, a polymer which was also found in high concentration in Sal estuary, Goa, as per a study conducted by Sahaa, et al., (2021).

4.2 Analysis of MPs (44µm to 0.22µm)

To verify the presence of microplastics, 40µl of suspension was smeared over each slide and air dried (Wang S. H., 1941). The slides were stained using Nile Red for 30 minutes and observed under light microscope using 10x objective, as shown in Fig 44A and 45A. The same slide was observed under epifluorescence microscope in which the background appeared dark and only particles emitted fluorescence as shown in 44B, confirming the identity of the particle to be a microplastic. Thus, counting of MP particles of the sample was done using epifluorescence microscope. Based on the microplastic count obtained using direct smear method for 500µl of the sample, result was estimated for the remaining 150ml of the sample. The different shapes and fluorescence exhibited by the particles are presented in Table 6 and depicted in Fig 46-53.

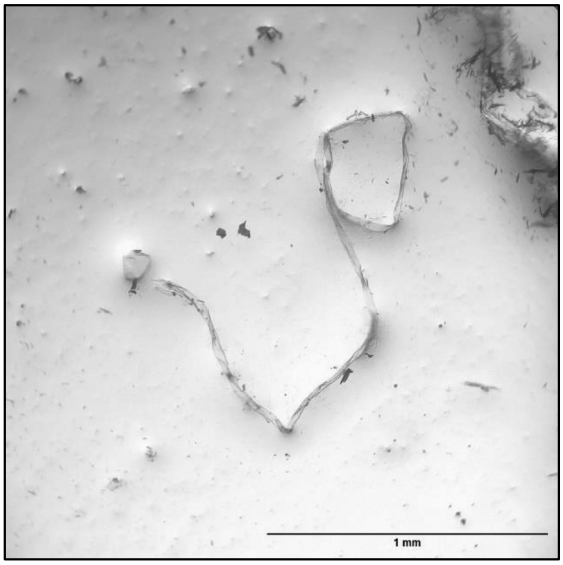


Fig 44A

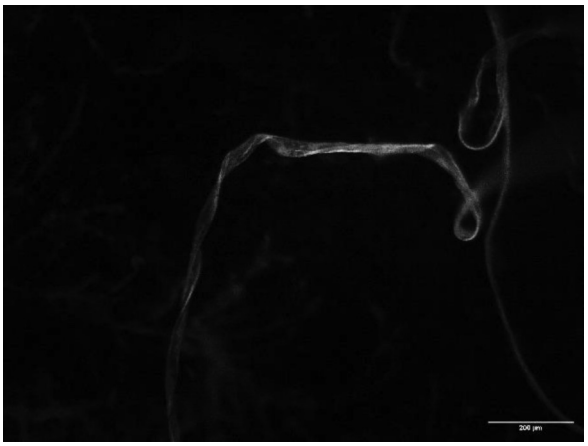
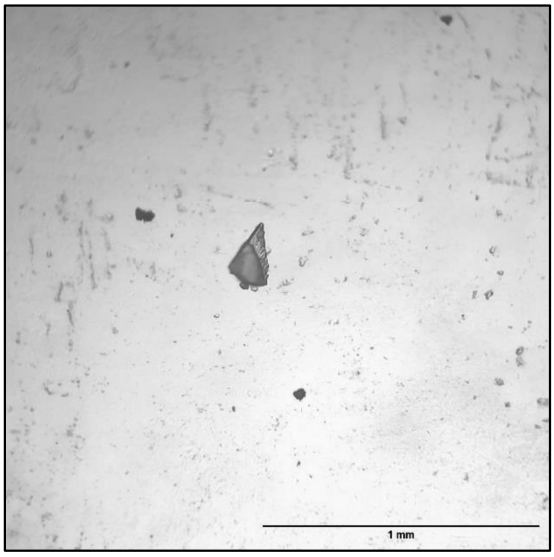


Fig 44B



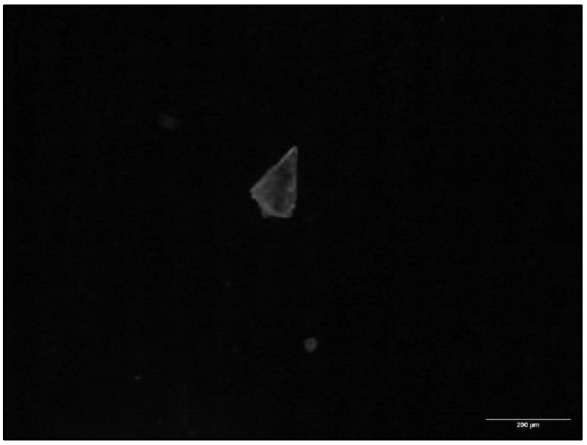


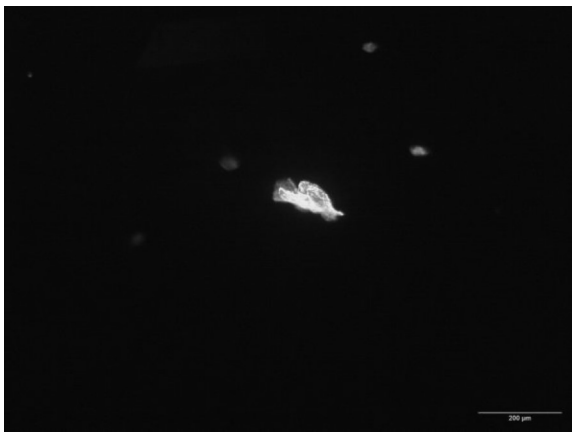
Fig 45 A

Fig 45B

4.3 Epifluorescence microscopic analysis

Table 6: Shapes and fluorescence emitted by particles in blue light (420-495nm)

Shapes of MPs particles	Fluorescence exhibited
Fibers, fragments, circular particles	Red, green, pink



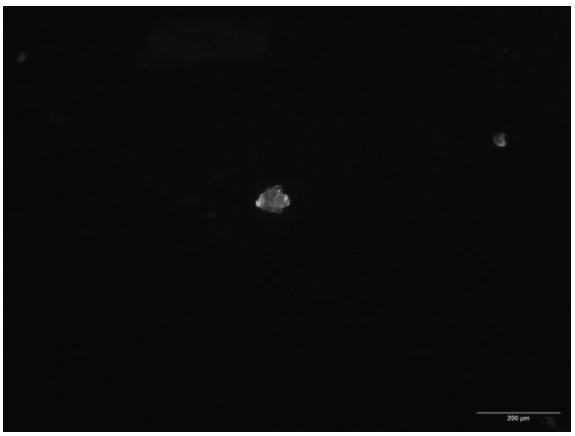
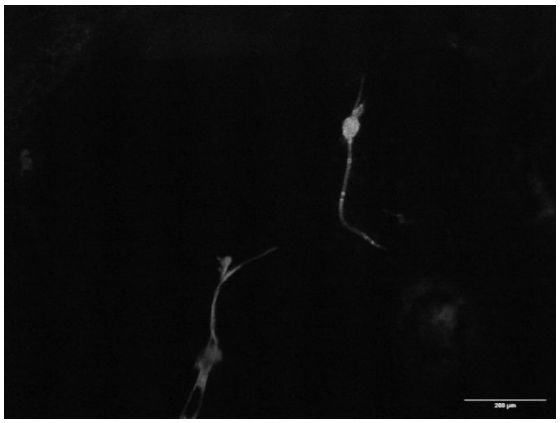


Fig 46

Fig 47

Fig 46-47: Microplastics showing green fluorescence under blue light



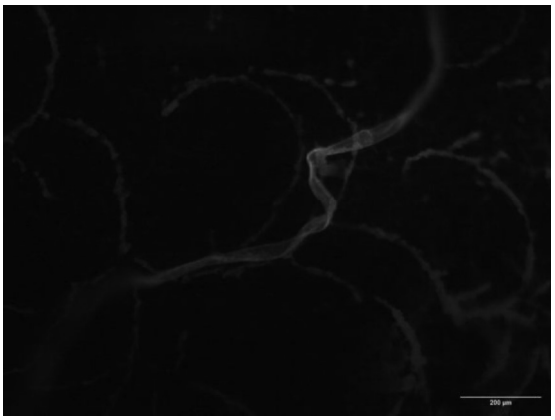
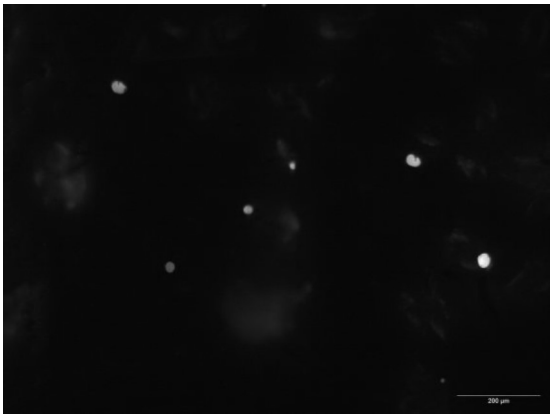


Fig 48 Fig 49

Fig 48-49: Microplastics fibres showing green and red fluorescence



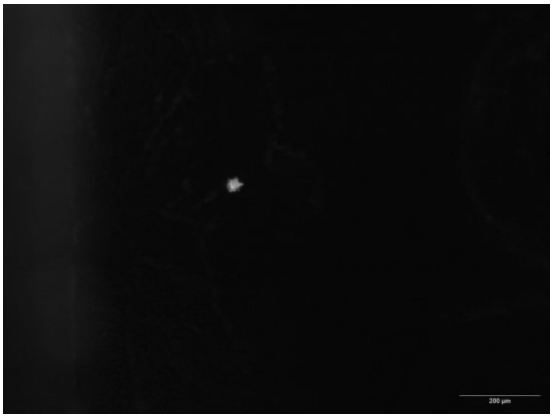
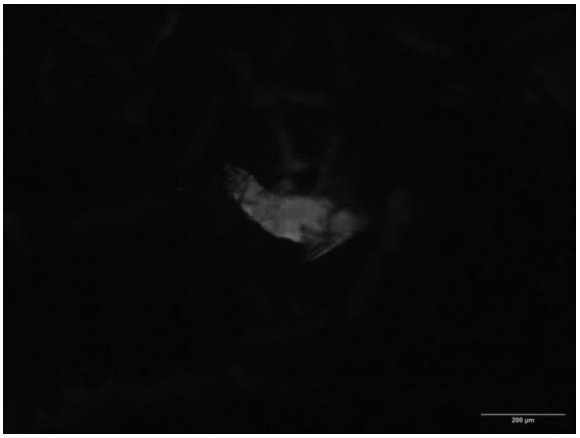


Fig 50: Spherical particles in blue light Fig 51: Microplastic showing pink fluorescence



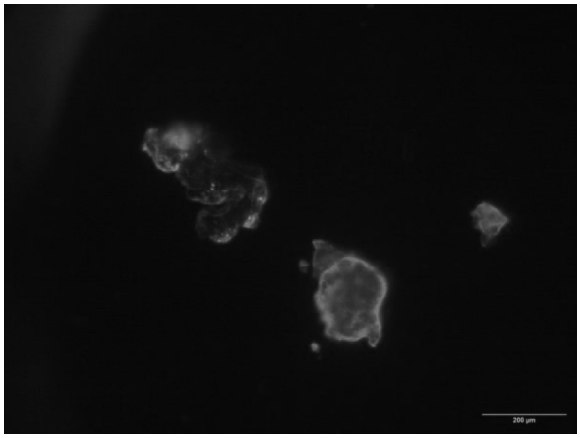


Fig 52 Fig 53

Fig 52-53: Microplastics showing red fluorescence

Fig 54

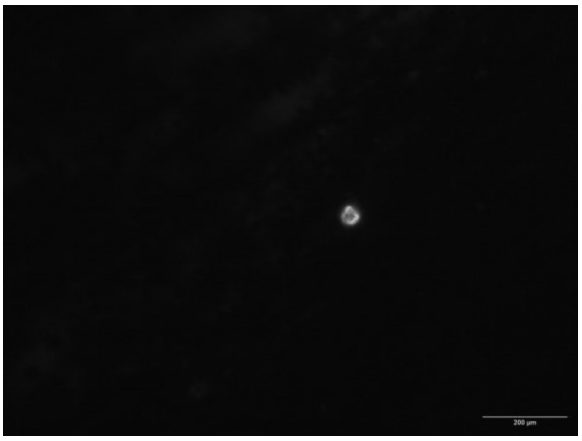


Fig 55

Fig 54-55: Microplastics on nucleopore membrane showing fluorescence under blue light

The total number of MP particles counted as counted under epifluorescence microscope was 189 in 500µl of the clams sample. The most frequently observed shapes of microplastics were fibres, fragments and spherical particles as shown in Fig 50. These results are in accordance with Wang, Li, & Wang, (2021) in which they found abundant fibrous and spherical MPs in the shellfish of the Jiangsu coastal region of China. The fluorescence emitted by these microplastics varied from red, pink to green, indicating that both polar and non-polar plastics are present in the sample. According to Gao, Wontor, & Cizdziel, (2022) polar polymers and non-polar polymers were shown to emit red and green fluorescence respectively. Comparing the results obtained from Raman spectroscopy of the larger microplastics, it can be inferred that the particles showing fluorescence in red range belong to categories of PM03 are polar polymers. While the particles emitting green fluorescence belong to PM01 and PM02.

The filtrate was further passed through 0.22µm nucleopore Whatman membrane and left overnight for drying. It was then stained using Rhodamine B dye and observed in epifluorescence microscope using 10x objective. The observed fluorescence by particles with RhB was similar to that of Nile Red, as shown in Fig 54 and 55.

4.3 Nanoplastic analysis (<200nm)

40µl of filtrate passed through 0.22µm was smeared over each slide and air dried (Wang S. H., 1941). The slides were stained using Nile Red for 30 minutes and observed under light microscope using 10x objective for particles having <200nm size.

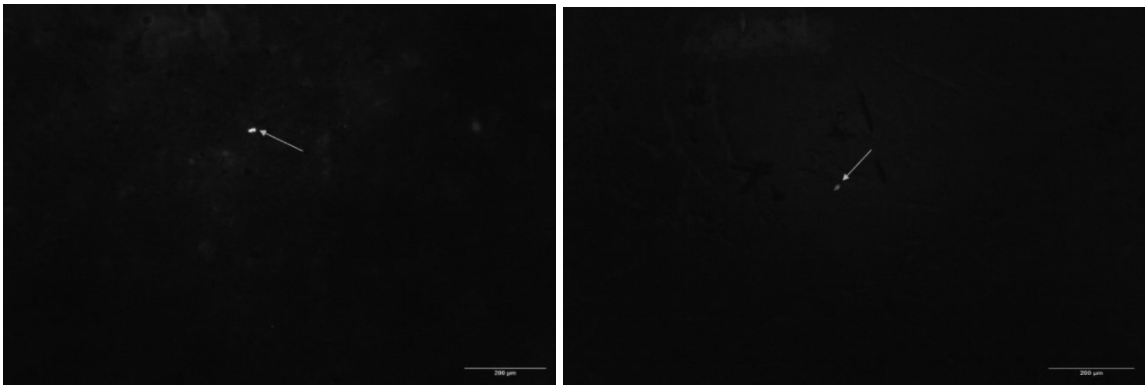


Fig: 56 Fig: 57

Fig 56-57 Nanoplastics showing different fluorescence in blue light

The particles (< 200nm) in clams sample emitted weak fluorescence ranging from red to green as seen in Fig. 56 and 57.

C] Positive control

Positive control for one of each polar plastic group was maintained. PET and PE were stained with NR and observed in blue light in epifluorescence microscope.

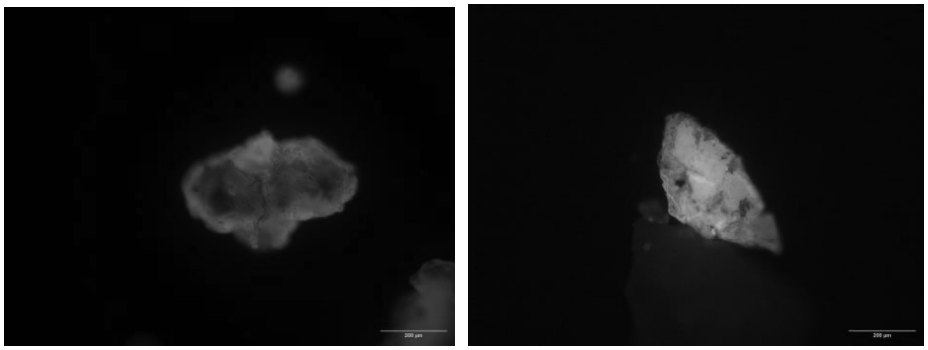


Fig. 58: Positive control for polar and non-polar MP in blue light

CHAPTER 5:

SUMMARY AND FUTURE PROSPECTS

5.1 Summary

In this study, assessment of microplastics and nanoplastics was carried out. Two different samples, *Gallus gallus domesticus* and *Paphia malabarica* were assessed for the presence of microplastics and nanoplastics using light microscopy, epifluorescence microscopy and μ-Raman spectroscopy. The analysis of each sample was based on three different size ranges, i.e., 4.9 mm to 45μm, 44μm to 0.22μm and size lesser than 200nm. The identity of these particles was done using μ-Raman spectroscopy and the spectrum similarity search done using KnowItAll Information System 2021 by Wiley Online Raman Database.

In *Gallus gallus domesticus*, 18 microplastics were isolated in the size range of 4.9 mm to 45μm which were classified based on visual characteristics. The polymers were identified as p-(acrylic acid), 4,4'-Ethylenedianiline, High Density Polyethylene and Polypropylene. According to results from epifluorescence microscopy, the dominant polymer shape in chicken gut was fragments and the most of them were polar in nature. Lastly, nanoplastics were detected as small dots showing weak fluorescence in epifluorescence microscope.

Similarly, from *Paphia malabarica* collected from Zuari estuary, 12 microplastics were isolated in the size range of 4.9 mm to 45μm which were classified based on visual characteristics. The polymers identified in clams sample as Polypropylene, Polyester, Polyethylene terephthalate and Polyethylene-covinyl-acetate. Different shapes of MPs were observed under epifluorescence microscope including fibers, fragments and spheres. The particles showed varying fluorescence intensities and colours, ranging from green to pink to red, confirming the presence of polar as well as non-polar MPs in the tissue of clams. Nanoplastics were detected as small dots showing weak fluorescence in epifluorescence microscope.

5.2 Future Prospects

- Isolation of microbial enzymes involved in plastic degradation
- Assessment of MPs and NPs in commonly consumed food items
- Study on toxicological effects of MPs and NPs in humans to combat associated health risks

CHAPTER 6:

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CHAPTER 7:

APPENDIX

7.1 Reagents preparation

7.1.1 10% KOH

10% KOH was prepared in a screw cap bottle by weighing 10g of KOH pellets and dissolving them in 100mL of distilled water. The bottle was marked as corrosive and stored in a cool, dry place.

7.1.2 Nile Red

Nile Red (NR) solution was prepared at 1 mg/mL in acetone in a clean 5ml vial. The vial was covered with aluminium foil and stored at room temperature away from light until use.

7.1.3 Rhodamine B

Rhodamine B (RhB) solution was prepared at 0.2mg/mL in ethanol in a clean 5ml vial. The vial was covered with aluminium foil and stored at 20˚C until use.

7.1.4 Epifluorescence microscope

7.2 Similarity search of plastic polymers done using Wiley Science Solutions's KnowItAll Raman Spectral Database Collection

Click on the new search in KnowItAll information system 2021 and add the spectra in .spc format. Start the search. The result page will list components that are similar to the given spectra and their percentage similarity. Choose the plastic polymer and this will give a graph comparing sample spectra to standard spectra. Use a snipping tool to cut and paste it word document.

Document Information

Analyzed document	Plagiarism check_033657.pdf (D164314665)
Submitted	2023-04-18 12:13:00
Submitted by	Veda Manerikar
Submitter email	biotech.veda@unigoa.ac.in
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