CHARACTERIZATION OF MICROPLASTICS AND NANOPLASTICS IN WATER, SEDIMENT AND BIOTIC SAMPLES AT VAGATOR BEACH

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DIVYANSHI SHARMA

21P050006

Under the supervision of

DR. SANJEEV C. GHADI

School of Biological Sciences and Biotechnology

Goa University

Date: April 2023

Seal of the School

Examined by:

sanitahotha
12/5/23

12/5/23

12/5/23

12/05/2023

DECLARATION

I hereby declare that the data presented in this Dissertation report entitled, "Characterization of micro plastic and nanoplastic in water, sediment and Biotic samples at Vagator Beach." is based on the results of investigations carried out by me in Marine Biotechnology at the School of Biological Sciences and Biotechnology, Goa University under the Supervision of Dr. Sanjeev 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 not be responsible for the correctness of observations / experimental or other findings given the dissertation. I hereby authorize the University authorities to upload this dissertation on the dissertation repository or anywhere else as the UGC regulations demand and make it available to any one as needed.

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Place- Goa University

Divyanshi Sharma
21P05006
Biotechnology Discipline
School of Biological Sciences and Biotechnology
Goa University

CERTIFICATE

This is to certify that the dissertation entitled "Characterization of micro plastic and nanoplastics in water, sediment and biotic samples at Vagator Beach" is a bonafide work carried out by Ms. Divyanshi Sharma under my supervision/mentorship in partial fulfillment of the requirements for the award of the degree of Masters in the Marine Biotechnology at the School of Biological Sciences and Biotechnology, Goa University.

Date:

Savitakerkar 12/5/23

Dr. Savita Kerkar
Marine Biotechnology
School of Biological Sciences and Biotechnology

Dean of School of Biological Sciences & Biotechnology
Goa University, Goa-403206
Office Ph. 8669609246

Dr. Sanjeev Ghadi
Marine Biotechnology
Dissertation Guide.

Place: Goa University

School Stamp

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LIST OF ABBREVIATIONS

Mm - Micrometer

Mm - Millimeter

Ml - Milliliter

Kg - Kilogram

G - Gram

Cm - Centimeter

℃ - Degree(s) Celsius

% - Percentage

< - Less than

MP - Microplastic

KOH - Potassium hydroxide

FTIR - Fourier transform infrared spectroscopy

SS - Sediment sample

PP- Polypropylene

PET- Polyethylene terephthalate

PBT- Poly butyl terephthalate

CONTENTS:

CHAPTER 1:
INTRODUCTION & LITERATURE REVIEW

INTRODUCTION

Plastic is a synthetic organic polymer derived from petroleum, commonly used in a wide range of applications such as packaging, building and construction, household and sports equipment, vehicles, electronics, and agriculture. Unfortunately, plastic pollution has become a pressing issue, with approximately 14 million tons of plastic ending up in the ocean each year. Plastic debris now constitutes the largest proportion of marine litter, accounting for up to 80% of all debris found in surface waters and deep sea sediments.

The 20th century witnessed a revolution in the plastic industry, leading to the manufacturing of a plethora of plastic-based products, from buckets to cars. However, the 21st century is now grappling with the consequences of plastic misuse, mismanagement, and lack of awareness about its detrimental effects. Improper disposal of plastic products has turned our planet into a "plastic planet," polluting not just roads, forests, and mountains, but also our oceans. Ignorant human behavior, driven by an "out of sight, out of mind" mindset, has resulted in plastic waste being dumped into water bodies, posing a severe threat to human and animal health (Sharma et al., 2021).

Plastics made from synthetic organic polymers can generally be classified into two types: thermoplastics and thermoset resins (gyer,2020). Thermoplastics are characterized by their reversible properties, meaning they can be melted when heated and hardened when cooled, allowing them to be reshaped into new materials through repeated heating and cooling cycles. Examples of thermoplastics include polyethylene (PE), polypropylene (PP), polyvinyl-chloride (PVC), polyethylene terephthalate (PET), polystyrene (PS), expanded polystyrene (EPS), acrylonitrile butadiene styrene (ABS), styrene acrylonitrile (SAN), polyamides (PA), polycarbonate (PC), poly methyl methacrylate (PMMA), and thermo-plastic elastomers (TPE). On the other hand, thermosets exhibit irreversible properties and cannot be melted or reshaped after the initial heating, due to a chemical change that occurs when heated. Examples of thermosets include polyurethane (PUR), unsaturated polyester, epoxy resin, silicone, and phenol-formaldehyde (PF) (Sharma et al., 2021).

TABLE 1 Classification of particulate plastics based on size:

Current size categories	Size range	Proposed size categories	Size Range	Organism of equivalent size
Nanoplastic	0.001-1 µm	Femto-size plastics	0.02-0.2 µm	Virus
Microplastic	1-1000 µm	Pico-size plastics	0.2-2 µm	Bacteria
		Nano-size plastics	2-20 µm	Flagellates
		Micro-size plastics	20-200 µm	Diatoms, dinoflagellates, ciliates, daphnids
		Meso-size plastics	200-2000 µm	Amphipods, appendicularians, chetognatos, copepods, thaliaceans
Mesoplastic	1-10 mm			
		Macro-size plastic	0.2-20 cm	Euphausiids, heteropods, jellyfish, larval fish, mysids, pteropods, solitary salps
Macroplastic	> 1 cm			
		Mega-size plastic	20-200 cm	Jellyfish, colonial salps

Microplastics (MPs) are defined as "synthetic solid particles or polymeric matrices, with regular or irregular shape and with size ranging from 1 μm to 5 mm, of either primary or secondary manufacturing origin, which are insoluble in water" (Frias & Nash, 2019).The adverse effects of microplastics on organisms can be categorized into two types: physical effects and chemical effects. Physical effects are related to the size, shape, and concentration of microplastics, while chemical effects are related to hazardous chemicals associated with microplastics (Laist, 1987).The coastline are where one third of the Indian population lives, including many communities dependent on fishing for their livelihood, is facing a global threat of plastic pollution due to lack of waste management strategies and discharge of wastewater into the ocean. India generates an estimated 25,903 t of plastic waste per day, or approximately 9.46 million t per year (Central Pollution Control Board-India, 2022).

A study by the National Institute of Oceanography (NIO) found that marine organisms along Goa's shoreline, specifically Vagator Beach in Bardez Taluka, Goa, are facing threats from the huge plastic debris that gets washed ashore during the monsoon season.

Seaweeds, as primary producers in the marine ecosystem, provide food and habitats for their consumers and other associated organisms. However, interactions of MPs with seaweeds and their fate in the marine food web are not well understood. One laboratory study showed that the periwinkle Littorina littorea, which feeds on the seaweed Fucus vesiculosus, can ingest both MPs and non-MPs contaminated algal food without any preference. This suggests that seaweeds can adsorb MPs and facilitate their transfer to organisms at higher trophic levels. Some species of seaweed, such as nori Pyropia spp., are also important ingredients in seafood and have close relationships with human health. In 2010, global seaweed production was 19 million tons with an estimated market value of 5.7 billion USD. China, the largest seaweed-producing country, accounted for about 60% of farmed seaweed yield by quantity and 45% by value worldwide. The red seaweed nori Pyropia spp. is an edible vegetable and easily processed, making it a commercially important seafood species in China. If the water environments used for nori cultivation are highly polluted by MPs, then MPs may contaminate and persist in nori even after processing and packaging.

GREEN MUSSEL: Perna viridis, also known as the Asian green mussel, is a commercially important bivalve belonging to the family Mytilidae. It is harvested for food and can reach lengths of 80 to 100 millimeters (3 to 4 inches), occasionally reaching up to 165 millimeters (6 inches). The shell of Perna viridis ends in a downward-pointing beak and has a smooth dark green periostracum that becomes increasingly brownish towards its point of attachment (umbo), where it is lighter. Younger mussels are bright green, which darkens as they age. The interior of the shell has a pale-blue sheen (Gobin et al., 2013). The mussel has a large mobile foot that it uses to climb vertically when covered by sediments, and it also produces byssus to attach to its substrate (Gobin et al., 2013).

It is important to provide a protocol for the separation and identification of microplastics in the marine environment that is easy to follow and adaptable depending on research infrastructure. Presented here is an optimized protocol that utilizes potassium hydroxide (KOH) for processing samples. KOH is a cost-effective, efficient, and simple alkaline digestant that allows for the extraction of plastics from the sample matrix. Samples are first digested, followed by filtration and selective fluorescent staining using Nile Red. The dye adsorbs onto plastic surfaces and renders them fluorescent when irradiated with blue light. Polymer identification is done using Raman spectroscopy.

LITERATURE REVIEW

MICRO-PLASTIC

Microplastics are small plastic particles measuring up to 5mm in diameter. They are fragments ofplastic that are less than 5 mm (0.20 in) in length, as defined by the U.S. National Oceanic and Atmospheric Administration (NOAA) (Collignon et al., 2014) and the European Chemicals Agency. The lower size limit of microplastics is not well-defined, but it is common practice to use the mesh size (333µm or 0.33mm) of the neuston nets used for sample collection (Arthur et al., 2009). The term "macroplastics" is used to distinguish microplastics from larger plastic waste, such as plastic bottles. Currently, two classifications of microplastics are recognized:

1. Primary Microplastic - Primary microplastics are intentionally created by manufacturers to be a specific small size for a particular purpose. They are small plastic spheres used in products such as face washes, cosmetics, and toothpastes for exfoliation or scrubbing. These spheres are typically made of polyethylene, polypropylene, polyethylene terephthalate, or nylon. Microbeads have received significant media attention recently, and some countries, including the US with the Microbead-Free Water Act of 2015, have banned their use. However, microbeads account for only 2% of the total microplastics released.

Microplastics are also generated as secondary microplastics through processes such as air-blasting technology. Small fragments of plastics, such as acrylic, melamine, or polyester, are blasted at high pressure onto machines, engines, or ship hulls to remove paint or rust. These fragments can become contaminated with heavy metals and are reused until they are no longer effective. Microplastics are also used in biomedical research for various techniques.

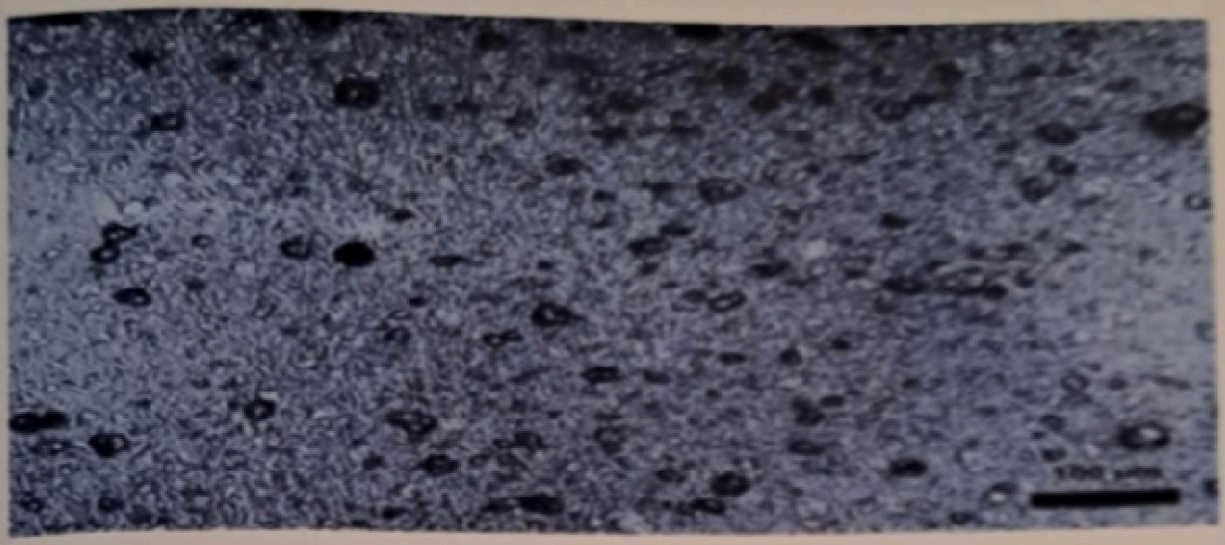

FIGURE 1: Polyethylene-based beads in toothpaste with a diameter of approximately 30 μm.

2. Secondary Microplastic - Larger pieces of plastic can degrade over time and release smaller fragments into the environment. Weathering, such as from waves, sunlight, or physical stress, can break down the plastic into smaller pieces. This type of microplastic usually originates from improperly managed waste, including plastic bags and fishing gear, which are common examples of garbage that breaks down. These microplastics often have varying shapes (Elena, 2023).

Given the significant amount of macroplastics entering the environment, it is generally believed that most microplastics in the environment are secondary microplastics (Andrady, 2011) (Hidalgo-Ruz et al., 2012) (Duis & Coors, 2016). Common polymers in the terrestrial environment degrade primarily due to UV radiation (Andrady, 2011) (Duis & Coors, 2016), resulting in plastic becoming brittle and eventually fragmenting. Factors such as high temperature, thaw-freeze cycles, and weathering can facilitate plastic degradation, leading to effective fragmentation on land and also on the beach surface.

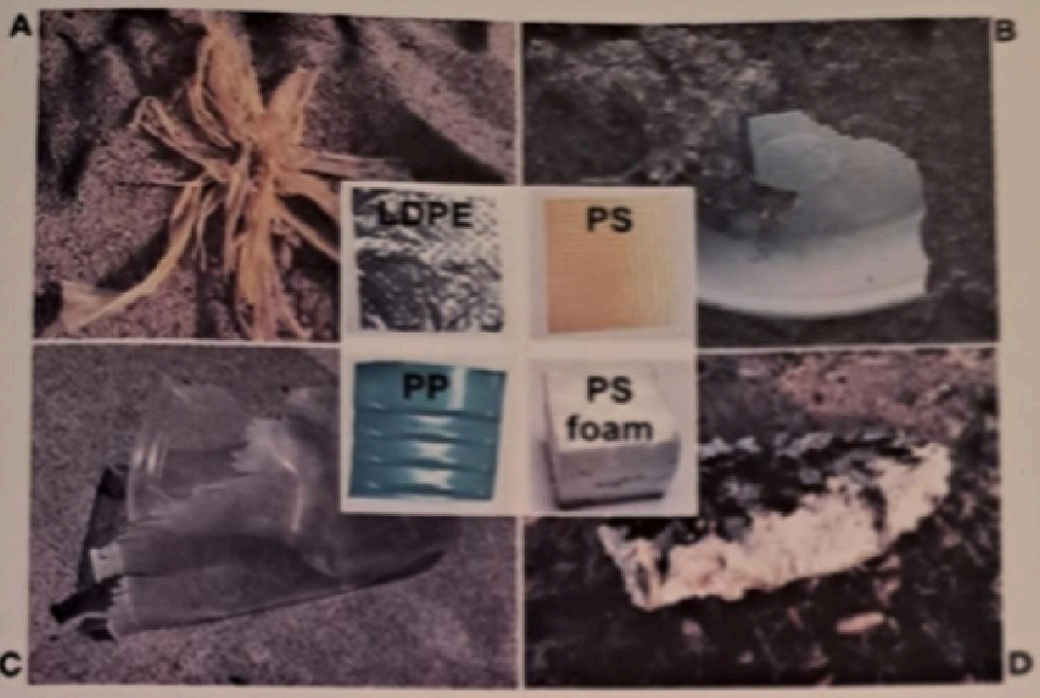

FIGURE2: Common type of plastic litter on the beaches of Baltic sea (A) polyethylene (LDPE) garbage bags, (B) polystyrene (PS) single-use plates, (C) polypropylene (PP) single-use beverage glasses, and (D) building insulations sheets (foamed PS)

3. In-Between Category

A third category exists between primary and secondary microplastics, which can be considered as an intermediate category. These microplastics are not intentionally designed to be of the size they are, but they do originate directly from human use, which leads to the argument that they should be classified as primary microplastics. These microplastics are derived from human use of objects that release microplastics, and unfortunately, it is more challenging to prevent their creation compared to making a conscious choice to avoid purchasing natural face scrubs.

Microplastics are classified based on their morphotypes or types of morphologies, such as fibers, fragments, films, pellets, beads, and Styrofoam. The abundance of microplastics in a water column depends on their density, where low-density microplastics like polyethylene (PE) and polypropylene (PP) tend to float in water, while high-density microplastics tend to sink in sediment (Ivar do Sul & Costa, 2014). They are further categorized based on the types of

polymers they are composed of, such as polypropylene (PP), polyethylene (PE), polystyrene (PS), nylon (N), polyethylene terephthalate (PET), polyvinyl chloride (PVC), and others.

PET belongs to the polyester polymer family and is synthesized from ethylene glycol and terephthalic acid. Due to its moisture and oxygen barrier characteristics, it is widely used in the food and beverage packaging industry (Lanaro et al., 2017). High-density polyethylene is a synthetic resin produced by polymerizing ethylene and is commonly used to manufacture blow-molded bottles, grocery bags, construction films, and more. PVC is produced by polymerization of vinyl chloride monomer using free radicals and is a widely used synthetic polymer after PE and PP (Kumar et al., 2022). Low-density polyethylene is polymerized from ethylene and is softer and more flexible compared to HDPE. PP is an olefin polymer with a low melting point, and it is widely used in carpets, sanitary products, surgical sutures, and more (Kourtidou et al., 2020). Polystyrene is an amorphous polymer, commercially known as crystal polystyrene, and possesses properties such as high clarity, colorlessness, and hardness (Niaounakis, 2015).

Sources of Microplastics

The presence of hazardous plastic fragments in the marine ecosystem is due to various anthropogenic activities, including domestic, industrial, and coastal activities. Microplastics are introduced into the aquatic ecosystem mainly through domestic runoff, which contains microbeads and microplastic fragments used in cosmetic and other consumer products, as well as from the fragmentation of large plastic waste (Andrady, 2011). Plastic manufacturing industries release plastics in the form of pellets and resin powders produced from air-blasting, which ultimately contaminates the aquatic environment (Claessens et al., 2011). Coastal activities such as fishing practices, aqua tourism activities, and marine industries are also sources of microplastic pollution in the marine ecosystem.

Most microplastic particles are composed of the six major polymer types. Microplastics composed of polyethylene, polypropylene, and expanded polystyrene are more likely to float, while those composed of polyvinyl chloride, polyamide (nylon), and polyethylene terephthalate (PET) are more likely to sink (Campanale et al., 2020). The surface of any solid object rapidly becomes coated with inorganic and organic compounds and biofilms when immersed in seawater, which may cause floating plastic particles to sink.

TABLE 2 Densities & Common applications of plastics found in the marine environment (Andrady,2011).

Resin Type	Common Applications	Specific Gravity
Polyethylene	Plastic bag, storage container	0.91-0.95
polypropylene	Rope, bottle, caps, gear, strapping	0.90-0.92
polystyrene	Cool boxes, floats, cups, utensils	0.01-1.05
Poly vinyl chloride	Film, pipes	1.04-1.09
polyamide	Fishing net, rope	1.16-1.30
Poly(ethylene terephathalene)	Bottles, strapping	1.34-1.39

Microplastics, once they enter the marine habitat, are exposed to various physical and chemical processes such as biofouling, leaching, or incorporation of secondary pollutants. They come in different shapes, sizes, and densities, and based on these characteristics, plastic fragments are distributed in different compartments of the marine ecosystem, eventually settling down to the benthos, and becoming available to marine biota (Sharma & Chatterjee, 2017). Objects with voids, such as bottles, tend to float initially, but once they lose their integrity, the density of the plastic determines whether they float or sink. The rate at which this occurs affects the distance the objects are transported from their source.

The distribution and abundance of microplastics in the world are extensive, and many scientists use them as key indicators of a new historical epoch known as the Plasticene. These synthetic polymers are environmental pollutants themselves and also act as vectors for transporting variouschemicals (Wagner, 2018). They are considered valid indicators of the recent and contemporary period, generally after the mid-20th century (Zalasiewicz et al., 2016). Nowadays, microplastic particles have been detected ubiquitously in a wide range of shapes, polymers, sizes, and concentrations in marine water, freshwater (Campanale et al., 2020), agroecosystems (Rillig et al., 2017), atmosphere (Prata, 2018), food (Waring et al., 2018), drinking water (Pivokonsky et al., 2018), biota (Rezania et al., 2018), and other remote locations (Cook, 2019). These tiny plastic fragments persist in the marine ecosystem and, due to their small size, are mistaken as food and ingested by various marine biota, including corals, phytoplankton, zooplankton, sea urchins, lobsters, fish, etc., and eventually transferred to higher trophic levels. The impact of microplastics on marine biota is a concern as it leads to entanglement and ingestion, which can be lethal to marine life. Microplastic fragments mainly come from terrestrial sources, posing a significant threat to coastal ecosystems, including coral reefs. Corals survive in a symbiotic association with single-celled algae that reside in the tissues of coral cavities. The algal association provides energy through photosynthesis, and corals also obtain energy by feeding onplankton to acquire essential nutrients for growth, development, and reproduction (Reichert et al., 2018). The "microplastic feeding" mechanism of corals involves ingestion, retention of plastic fragments, and digestion (Lusher et al., 2015). The harmful effects of microplastics on corals include the retention of plastic fragments in mesenterial tissue, leading to reduced feedingcapability and decreased energy reserves (Reichert et al., 2018).

Exposure to microplastics in laboratory settings has been shown to cause potential physical harm to species of various taxa, such as inhibition of digestive processes, abrasion, and lesions in the gut, and generation of a misleading sensation of satiation, resulting in toxic anorexia, ultimately leading to malnutrition and starvation, similar to the effects of macroplastics (Meyers et al., 2022). Although microplastics are often undigested, they can transfer chemicals adsorbed or added to their matrix to organisms, via desorption of co-contaminants or leaching of chemical additives, with the direction of chemical flux often depending on the trophic level of the organism (Koelmans et al., 2019).

NANOPLASTICS

We define nanoplastics as particles unintentionally produced (i.e., from the degradation and manufacturing of plastic objects) and exhibiting colloidal behavior, within the size range from 1 to 1000 nm (Gigault et al., 2018).

With the increasing use of the term "nanoplastic," the term "microplastic" can be considered outdated. Recently, a new concept has emerged in the scientific literature: micro(nano)plastics. This term is used to refer to both micro- and nanoplastics. However, we argue that the environmental fate and behavior of plastic particles of different sizes are distinct enough that they should be studied and described independently, rather than combined in the term micro(nano)plastics (ter Halle & Ghiglione, 2021). Furthermore, combining both notions in the term micro(nano)plastics is a shortcut that can lead us to overlook the fact that the transport, reactivity, and biological interactions of these particles are not the same (ter Halle & Ghiglione, 2021).

Examples of how nanoplastics differ significantly from microplastics include their behavior in environmental matrices. Our understanding of nanomaterial science suggests that nanoplastics in environmental matrices are typically coated with molecules to form an "eco-corona" (Monopoli et al., 2012). In parallel with nanotechnology, where engineered nanoparticles tend to aggregate into clusters up to several micrometers in size, it is expected that nanoplastics may also spontaneously aggregate and exist as large colloids a few hundred nanometers in size. Smaller plastic particles may then likely bind to these larger colloids. This dynamic process of

spontaneous nanoplastic heteroaggregation and disaggregation is a critical factor in determining the reactivity, toxicity, fate, transport, and risk to the environment and organisms (Monopoli et al., 2012).

Figure 3. Hypothesized interactions in the environment between nanoplastics, natural organic matter or ions, and natural colloids. A: Plastic particles on the nanoscale are coated with dissolved molecules, macromolecules, and ions, forming the eco-corona of the particle. The binding of the nanoplastic through its eco-corona with apolar molecules can lead to aggregation through the formation of bridges (cases B and C). B: Nanoplastic heteroaggregation with inorganic particles (such as clay). C: Nanoplastic heteroaggregation with organic colloids (particularly organic matter). Ions can also promote aggregation (bridging effect, cases D and E). D: Heteroaggregation with inorganic particles. E: Heteroaggregation with colloidal organic matter. The bridging effect is when ions (Ca^{2+}, Fe^{3+}, etc.) or inorganic colloids (Fe_2O_3) induce the formation of clusters between nanoplastics and natural colloids.

Another illustration of how the term micro(nano)plastic may be misleading is the association of this term with the plastisphere, that is, the microorganisms living on plastics.

Figure 4. Conceptual figures illustrating the interactions between microorganisms and plastic particles. A: Microplastic (a few millimeters long) colonized by microorganisms that form a biofilm on its surface, called "plastisphere". In nature, the plastisphere is an inhomogeneous biofilm covering parts of the microplastic. B: Conceptualization of the interactions between a bacteria (a few micrometers long) attached to nanoplastics in its most likely state of heteroaggregate in the environment (a few hundred nanometers long). Not drawn to scale.

Methods for isolation and identification of micro & nano plastics.

Sample preparation is necessary to separate the microplastics from the sample matrix. The fish sample is dissected to remove gastrointestinal tract (Pradit et al., 2021). Digestion of natural organic material is commonly done using hydrogen peroxide (30% or 50%) with Fe II as a catalyst or potassium hydroxide KOH (10%), concentrated hot nitric acid HNO3[45]. Digestion is done at 60˚C for complete digestion of organic matter . Sediment sample are separated/extracted using density gradient utilizing saturated salt solution (NaCl) that results in floating of lower density polymer particles on the top of the solution (Halfar et al., 2021). Filters like glass-fiber, stainless-steel, or membrane filters are used for processing the sample before analyzing it through microscopy or spectroscopy . The mesh size of the filters can vary widely depending on the desired size of the MP to be analyzed. Once the MP are isolated, they are analyzed using spectroscopic or microscopic methods. Characterization of isolated microplastic

is done on the basis of shape, size, color, and polymer. The shapes/forms of MP are classified as films, fragments, fibers, pellets, and foam.

Based on literature, the commonly used analytical instrumental methods are Fourier transform (FTIR), Raman spectroscopy (RS), or scanning electron microscopy (SEM). Other possible methods or combination of methods that can be used are Pyrolysis-gas chromatography/mass spectrometry (Pyrolysis-GC/MS), thermogravimetry with differential scanning calorimetry (TGA–DSC), liquid chromatography with mass spectroscopy (LC/MS) or energy dispersive X-ray spectroscopy (EDS) (Halfar et al., 2021). Mendoza et al (2019) had recommended the use of spectroscopy (ATR-mFT-IR and mRAMAN) and Pyrolysis-Gas Chromatography coupled to Mass Spectrometry for quantitative identification of various types of polymers in MP. The surface morphology and characteristics are investigated with SEM, and polymer identification is done using Raman spectroscopy and/or FTIR (Yin et al., 2019) (Koelmans et al., 2019). The various types of polymers that are identified by most researchers while assessing the MP are Polypropylene (PP), polyethylene (PE), polystyrene (PS), polyethylene terephthalate (PET), polyamide (PA), and polyvinyl chloride (PVC). In most of the studies Polypropylene was found to be dominant (Yin et al. 2019). MP being very small in size pose a challenge to differentiate them from natural debris, therefore assessing their polymer type, size, and form plays a major factor in distinguishing them. To date, there are only five studies reporting the detection of native nanoplastics using Raman spectroscopy or pyrolysis gas chromatography mass spectrometry (Py-gc-ms). In addition, these new nanoplastic detection techniques do not allow robust quantification of native nanoplastics. Nanoplastic particles are highly reactive and easily destabilized or aggregated, which can lead to substantial losses during sample preparation. These losses have not yet been quantified, preventing reliable quantification of nanoplastic presence. In contrast, microplastic methods of quantification have been more extensively evaluated and discussed.

Monitoring of MPs in various biotic and abiotic environmental matrices is necessary to define the state of pollution, flow, and risk of exposure by organisms. Monitoring studies require reliable and comparable methods. he Nile Red staining method does not reach the reliable quantification capabilities of Raman-spectroscopy or Fourier transform infrared spectroscopy. Nevertheless, it can aid the assessment of microplastic abundances. In conclusion staining with

Nile Red does not require expensive equipment and allows the quick evaluation of a large number of samples for the assessment of microplastics. In a study, 1 g of marine sediment (SPI6) spiked with microplastics of six different polymer types, dyed with Nile Red (1000 µg/ mL ,30 minutes), then filtered onto a 47 mm diameter membrane filter.

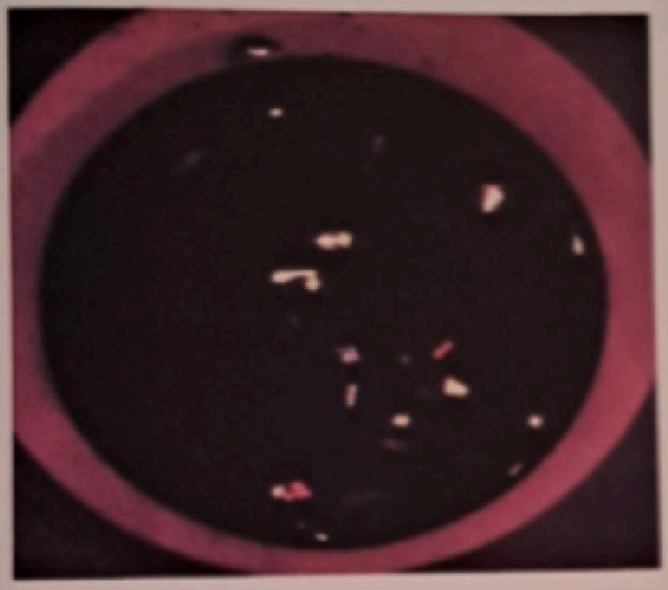

FIGURE 5: Photograph of filter in blue light.

From these initial tests, it was also apparent that the different types of plastic displayed differentfluorescent colours when stained with NR.

CHAPTER 2

AIM AND OBJECTIVES

: Aim

- To assess the presence of microplastics and nanoplastics in surface water sample, sediment sample, *Ulva fasciata*, *Perna viridis*.

: Objectives

- Isolation of microplastics and nanoplastics from surface water , sediment , *Ulva lactuca* (seaweed sample), *Perna viridis*(green mussel).

- Characterization of the isolated microplastics and nanoplastics in surface water , sediment , *Ulva fasciata (seaweed), Perna viridis* (green mussels)

- Identification of the type of polymer of the characterized microplastic and nanoplastic in surface water , sediment , *Ulva fasciata* (seaweed), *Perna viridis* (green mussel)

CHAPTER 3

MATERIALS AND METHODS

3.1.Sample collection and measurement:

3.1.1 Sampling Site: **Latitude:** 15° 35' 51.58" N

Longitude: 73° 44' 41.86" E

All the 4 samples: surface seawater, sediment, *Ulva fasciata* (seaweed), *Perna viridis* (greenmussel) were collected from Vagator beach.

FIGURE 6: Sampling site (Vagator beach)

3.1.2 Water Sample: One liter surface water sample was collected in glass container from the sampling site. Temperature and pH were checked on site.

FIGURE 7: Surface water sample

3.1.3 Sediment sample: One kilogram of sediment sample was collected in glass container fromthe sampling site. Temperature and pH were checked on site.

3.1.4 *Ulva fascinata*: Samples can be selected at random from the subtidal area of 0.5 to 3 m. seaweedspresent in the quadrant are collected in polyethylene bags. Seaweed is weighed on a weighing scale.

Figure 8: Seaweed Sample (Ulva lactuca)

3.1.5 *Perna viridis:* 2 green mussels were selected for studying and weighed on a weighing scale.

Figure 9: Green mussel (*Perna viridis*)

3.2 Preparation of sample and isolation of microplastic and nanoplastic:

3.2.1 Surface water sample- One liter surface water sample was taken and sieved using four sieves having pore sizes of 5 mm, 0.5mm, 0.2mm, 0.044mm. The microplastics that were visibleon the filter paper were isolated, and magnifying glass and microscopes were used to check the smaller microplastic particles. The remaining water sample that passed through the sieve was collected and filtered using 0.2µm size whatman membrane filter. We prepared 3 slides containing 40µl each using this filtrate and let it dry for 5 minutes and then stained with fluorescent dye Nile Red (NR). The slides are incubated at room temperature for 30 minutes. After that the slides are observed under fluorescence microscope using blue light.

3.2.2 Sediment Sample - One kilogram of sediment sample was taken and allowed to dry completely in a hot air oven at 60˚C. Once the sample was completely dried, it was sieved using four sieves having pore sizes of 5mm, 0.5mm, 0.2mm, and 44 µm. Microplastics were isolated from the sieves using forceps. Magnifying glass and microscopes were used to check the smallermicroplastic particles, these were then collected using fine forceps.

3.2.3 *Ulva fasciata*: The seaweed sample is first weighed using a weighing scale and then put into a glass beaker and allowed to dry in hot air oven at 60˚C overnight to remove all the water from the seaweed. The dried seaweed is weighed again using a weighing scale. A triple amount of 10% of KOH was added to the sample for organic matter digestion. It was kept at 60˚C in a hot air oven for 2 days or more till organic matter digested completely. It took around 20 – 25 days (about 3 and a half weeks) for seaweed to completely digest. The digested sample was sieved using four sieves having pore sizes of 5 mm, 0.5mm, 0.2 mm, 0.04mm. Microplastics were isolated from the sieves using forceps. Magnifying glass and microscopes were used to check the smaller microplastic particles, these were then collected using fine forceps in a petri plate, which were later sent for micro FTIR analysis. The remaining filtrate was passed through 0.2µm size whatman membrane filter. We prepared 3 slides containing 40µl each using this filtrate and let it dry for 5 minutes and then stained with fluorescent dye Nile Red (NR). The slides are incubated at room temperature for 30 minutes. After that the slides are observed underfluorescence microscope using blue light.

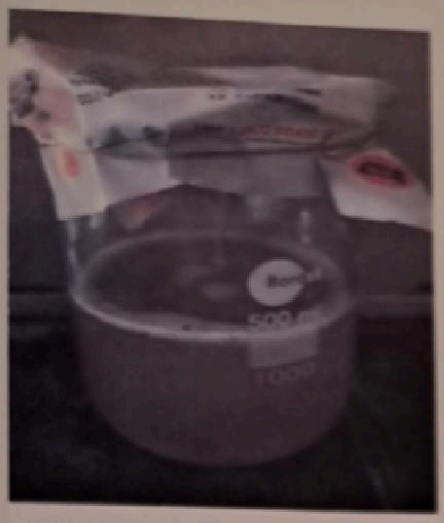

FIGURE 10: Digested seaweed sample

3.2.4 Perna viridis: The green mussel sample is first weighed using a weighing balance and then put into a glass beaker. A triple amount of 10% of KOH was added to the sample for organic matter digestion. It was kept at 60°C in a hot air oven for 2 days till organic matter digested completely. The digested sample was sieved using four sieves having pore sizes of 5mm, 0.5mm, 0.2 mm, 0.04mm. Microplastics were isolated from the sieves using forceps. Magnifying glass and microscopes were used to check the smaller microplastic particles, these were then collected using fine forceps in a petri plate, which were later sent for micro FTIR analysis. The remaining filtrate was passed through a 0.2µm whatman membrane filter. Slides were prepared containing 40 µl of this filtrate and let it dry for 5 minutes and then stained with fluorescent dye Nile Red (NR). Slides were incubated at room temperature for 30 minutes. After that the slides are observed under fluorescence microscope using blue light. Around 500 µl of filtrate was analyzed using this technique.

FIGURE 11: Digested mussel sample

FIGURE12: Sieves used for filtering microplastics.

FIGURE 13: Filteration using 0.2µm whatman filter paper

3.4Washing of microplastic:

Isolated microplastics were washed/cleaned with 100% ethanol and were allowed to air dry.

3.5 Characterization of isolated microplastic:

Microplastic were Characterized on the basis of texture, transparency, hardness/softness, color.

3.6Microscopic analysis for scaling of isolated microplastic:

Isolated microplastics were placed onto the slide, observed under 10x objective, and images were taken of the same. Microscopic scaling was done using ImageJ software using a hemocytometer as the reference.

3.7 Micro Raman spectroscopic analysis:

Each category of microplastics was given a code name based on the type of sample. Microplastics from water samples were given code names with the prefix WS which is denoted as Water Sample. Microplastics from Sediment sample was given code name with the prefix SS.Microplastic from Seaweed sample given code name with the prefix UL. Similarly, microplastic from *Perna canaliculum* (green mussel) was given a code name MS denoting Mussel Sample.

Search for isolated particles was done using KnowItAll Information System 2021 by Wiley Online RAMAN Database.

3.7. Flourescence microscopic analysis and scaling

Fluorescence staining methods provide a simple and sensitive approach to highlighting specific objects or structures in biological and medical studies. Nano plastic counting and identification is done with selective fluorescent staining using Nile Red (NR) for plastic particles smaller than 0.2µm in size. The dye adsorbs onto plastic surfaces and renders them fluorescent when irradiated with blue light. Fluorescence emission is detected using simple photography through an orange filter. Image-analysis allows fluorescent particles to be identified and counted. The solvatochromic nature of Nile Red also offers the possibility of plastic categorization based on surface polarity characteristics of identified particles.

CHAPTER 4
RESULTS AND DISCUSSION

4.1.Microplastic Assessment :

4.1.1.Water Sample

Microplastics were observed on the sieve with pore size 5mm, 0.5mm, 0.2mm & 0.04mm and were collected on petri plate using forceps. Visualcharacterization of microplastics was done based on color, thickness, transparency, hardness, shape. Based on which the isolated microplastics were goruped in different classes with code WS.

Table 3 Visual Characterization of Microplastic isolated from surface water sample:

Designated MP	Total no. Of microplastic particles	color	Thickness	transparency	hard/ soft	weight (mg)	Shape
WS01	4	–	thin	transparent	soft	0.2	fragment
WS02	1	green	thin	opaque	hard	0.2	film

Isolated microplastics were washed using 100% ethanol to remove the soil particles settled on them. But they were not clear enough even after washing it twice with 100% ethanol. Most microplastics were seen to be transparent in appearance. Even though the characteristics were observed to be similar, microplastics of different categories visually looked different.

1Liter of surface water sample contained 5 particles of microplastics.

41.1.1.Microscopic analysis and microplastic scaling

Images depicting the microplastics from water sample with scale under the microscope:

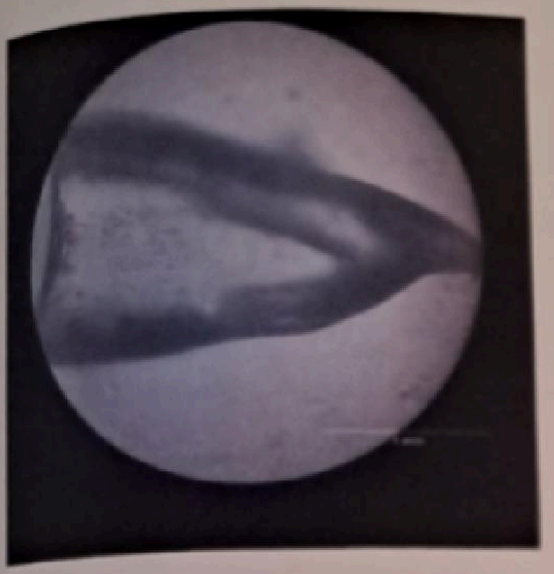

Figure 14: WS01

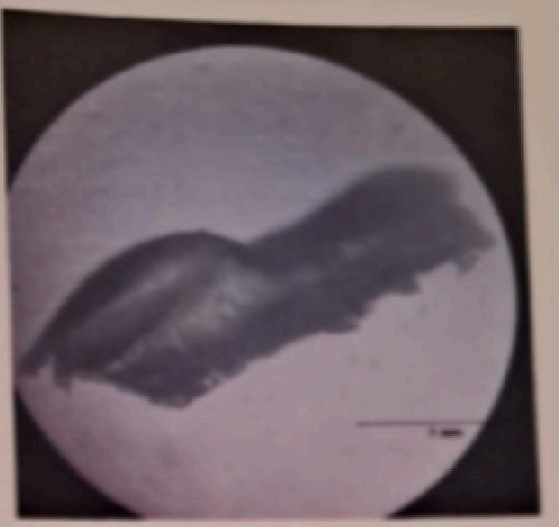

Figure 15: WS02

4.1.1.2.Raman Spectra with Standard plastic polymer spectrum for microplastics in surface water samples: (All spectrum above 70% similarity were chosen)_

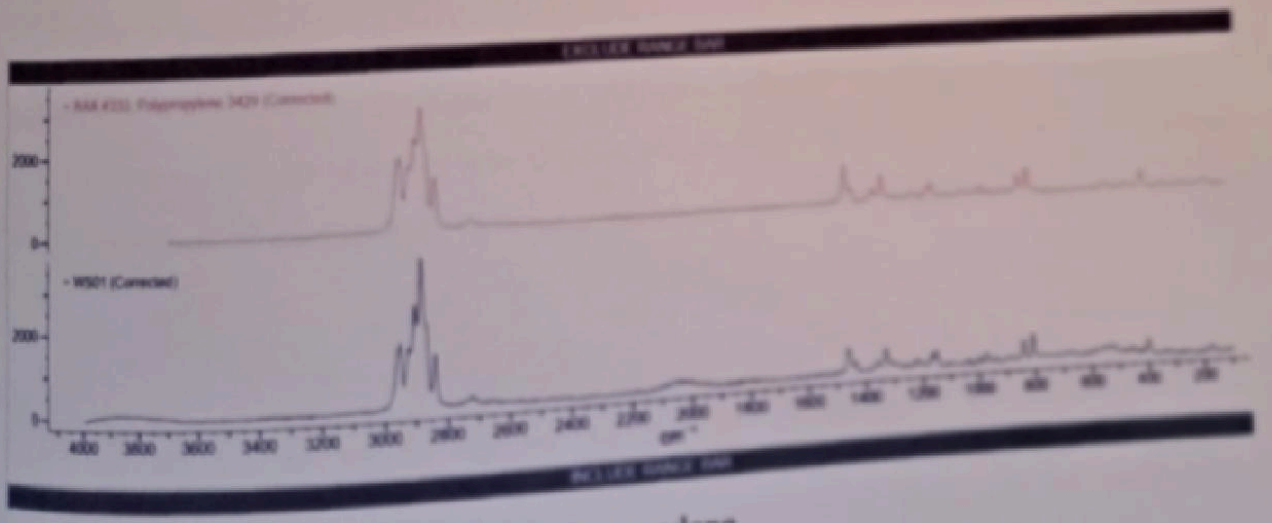

FIGURE 16: Raman spectrum for polypropylene.

Microplastics in class WS01 showed similarity to polypropylene

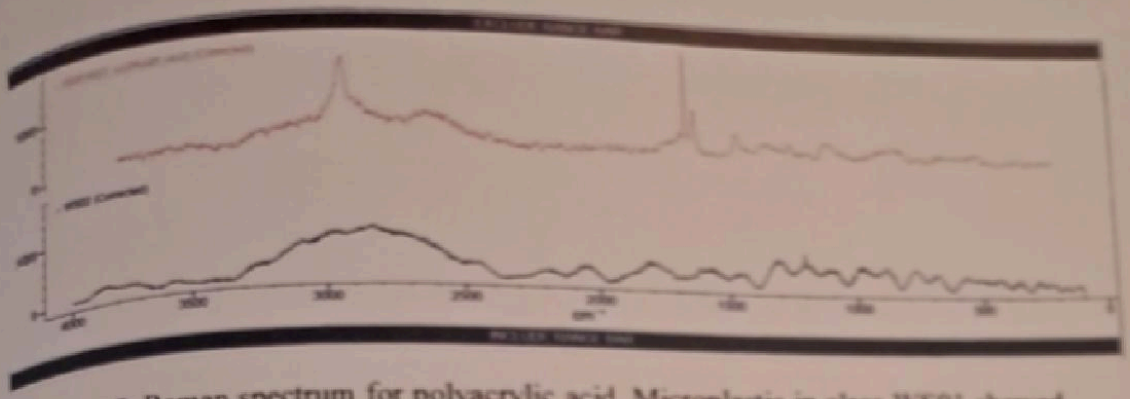

FIGURE 17: Raman spectrum for polyacrylic acid. Microplastic in class WS01 showed similarity to polyacrylic acid.

The water sample was mostly clear and contained a little amount of microplastic. From 1 liter of water sample 5 microplastic particles were observed. After visual characterization of microplastics they were classified into 2 classes. One microplastic from each class was scaled using light microscopy with hemocytometer as the reference and was further given for micro-Raman spectroscopy analysis. They were mostly in the form of a fragment or film. This result was in accordance with the study conducted in goa. According to the paper water samples showed a slight dominance of fibres over fragments which was followed by films (Saha et al., 2021). Although no fibers were observed and the microplastic isolated was smaller than what was found in similar studies. Polypropylene was the major microplastic isolated from surface water sample followed by polyacrylic acid. This result was in accordance with studies done by Gabriel et al. (2019) which states that lower density polymers, such as polypropylene, dominated sea surface samples.

4.1.2 Sediment sample:

Microplastics were observed on the sieve and were collected on petri plate using forceps. Visual characterization of microplastics was done based on color, thickness, transparency, hardness, shape. Based on which the isolated microplastics were grouped in different classes with code SS.

Table 4 Visual Characterization of Isolated Microplastic in Sediment Sample;

Designated MP	total no. of microplastic particles.	color	thickness	transparency	hard/ soft	weight (mg)	Shape
SS01	1	white	thin	opaque	Soft	1.1	Fragment
SS02	2	green	thick	opaque	Hard	2.1	Fragment
SS03	3	blue	thin	opaque	Hard	0.1	Fragment
SS04	6	white	thin	opaque	Soft	0.6	thread like
SS05	4	_	thin	transparent	Soft	0.5	film like

1 Kg of Sediment Sample contained 16 microplastic particles.

4.1.2.1 Microscopic analysis and microplastic scaling

Images depicting the microplastics from sediment with scale under the microscope:

Figure 18: SS01

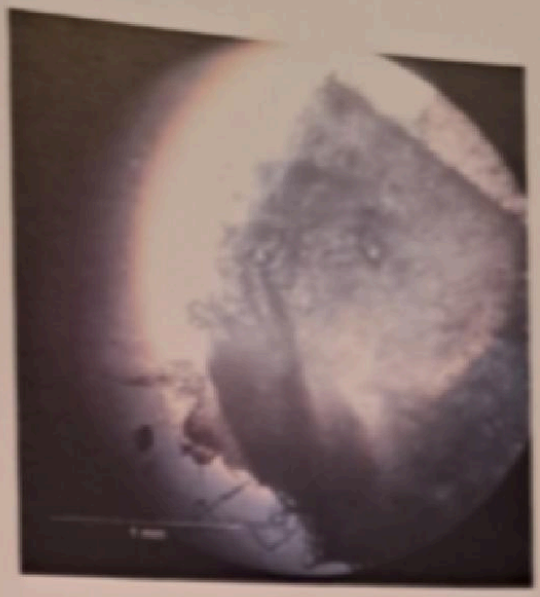

Figure 19: SS02

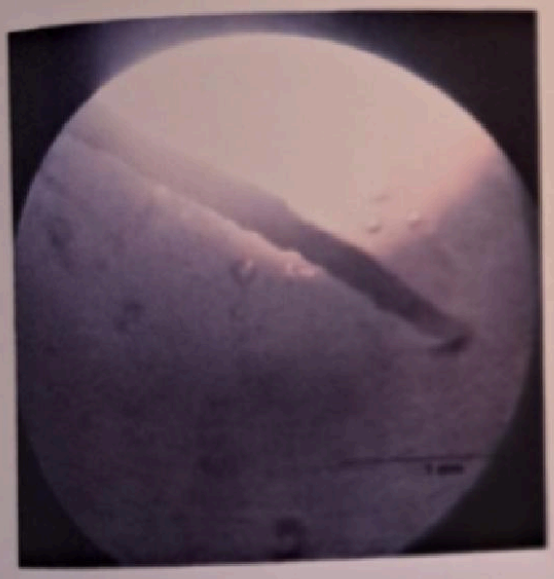

Figure 20: SS03

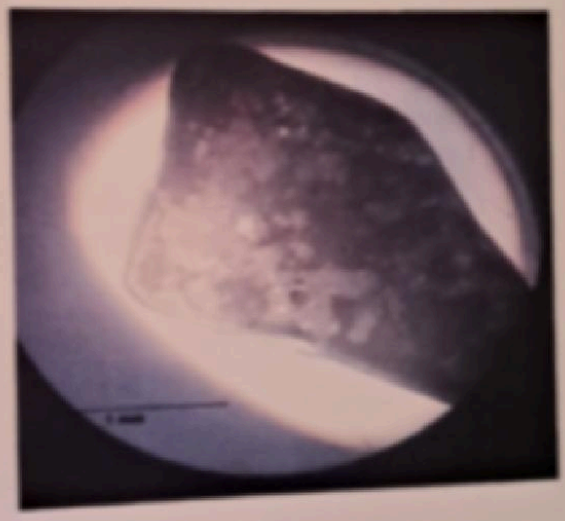

FIGURE21: SS04

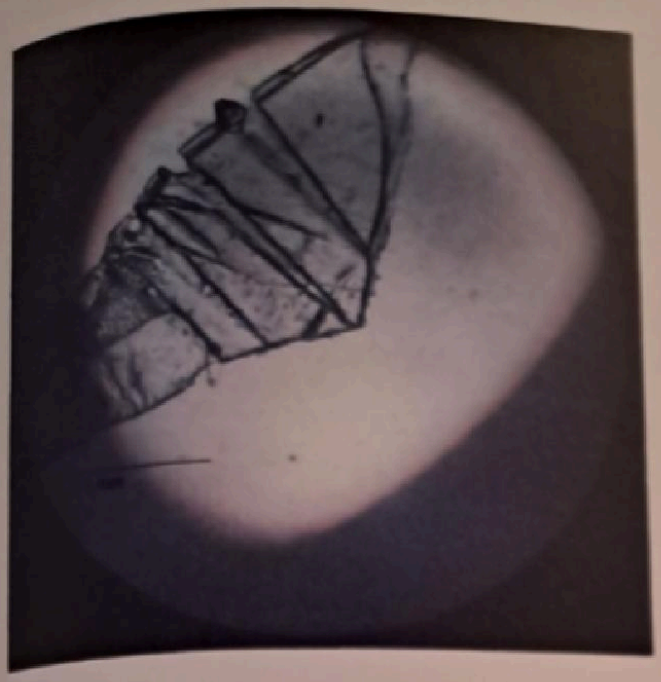

Figure 22: SS05

4.1.2.2. Raman Spectra with Standard plastic polymer spectrum for microplastics in sediment sample: (All spectrum above 70% similarity were chosen)_

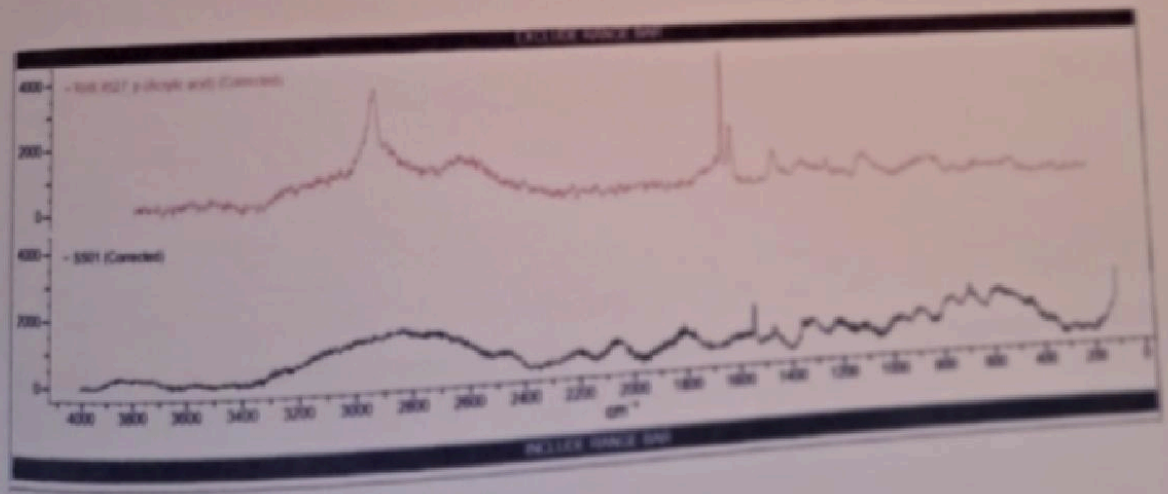

FIGURE 23: Raman spectrum for poly acrylic acid.

Microplastic in class SS01 showed similarity to poly acrylic acid.

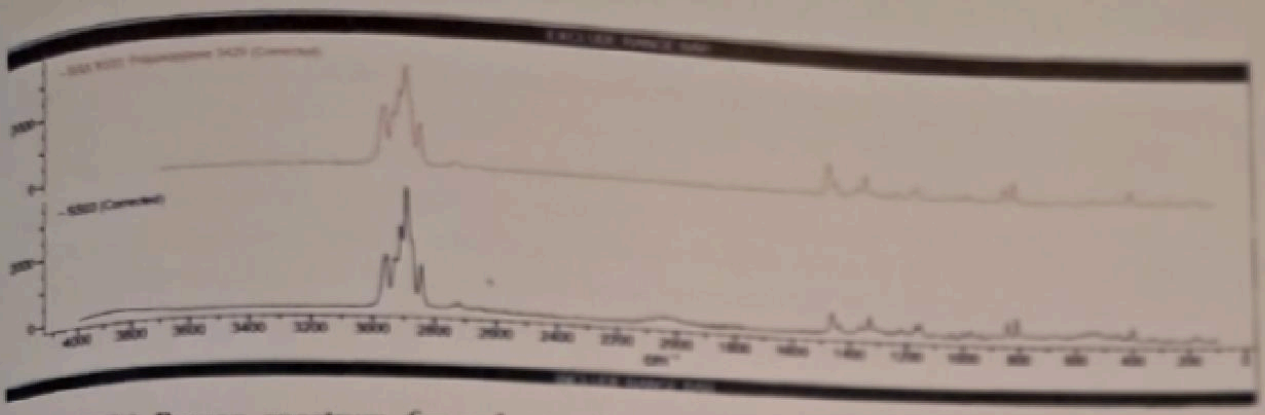

FIGURE 24: Raman spectrum for polypropylene.

Microplastic in class SS03 showed similarity to polypropylene

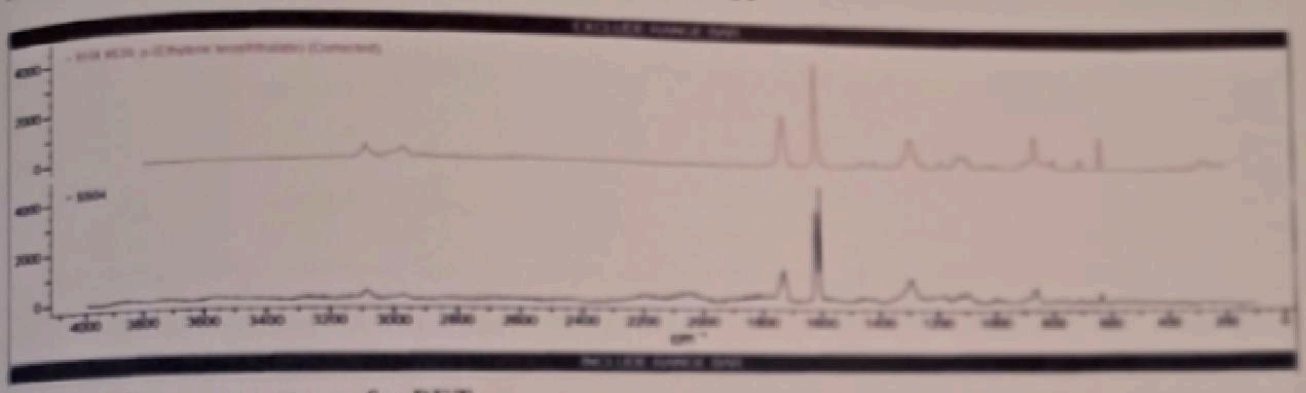

FIGURE 25: Raman spectrum for PET.

Microplastic in class SS04 showed similarity to PET.

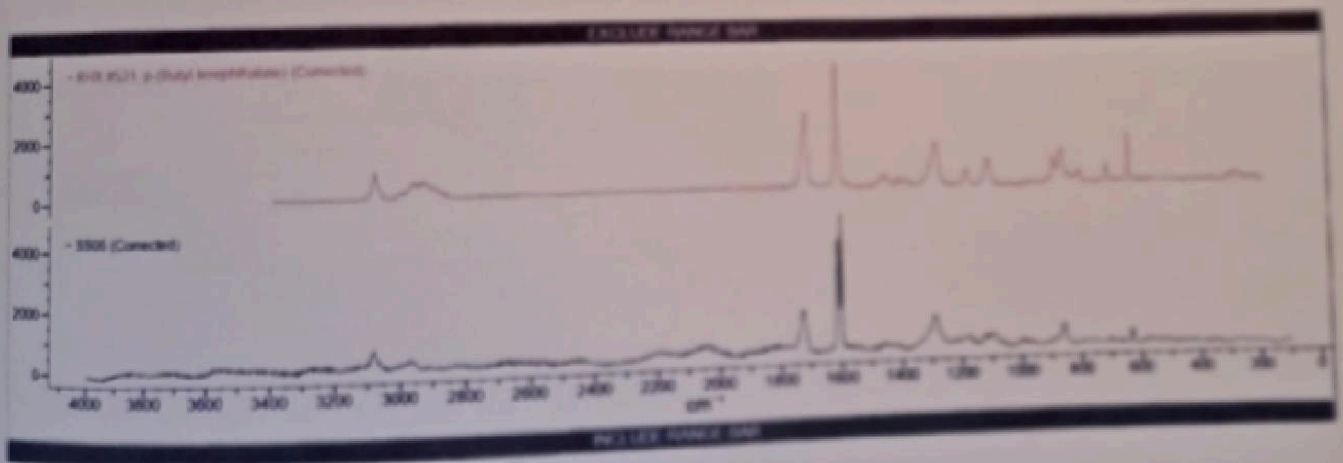

FIGURE 26: Raman Spectrum for PBT.

Microplastics in class SS05 showed similarity to PBT.

From the Sediment sample, 16 microplastic particles were isolated and classified into 5 classes.After visual characterization of microplastics they were classified into 2 classes. One microplastic from each class was scaled using light microscopy with hemocytometer as the reference and was further given for micro- Raman spectroscopy analysis. Many of them were threads and fragments. This was in accordance with the study conducted in goa in which majority of microplastics were threads in sediment sample (Saha et al., 2021). In sediment sample, PET was found to be the most abundant microplastic. The second prominent microplastic polymer was PBT followed by polypropylene. In a study conducted at Vietnam, atCan Gio beach, researchers found PET to be second most abundant microplastic in sediment sample (Khuyen et al., 2021). Polypropylene was also reported in high quantities in similar studies. polypropylene products are popular as disposable bottle caps, cups and straws.

4.1.3. Seaweed Sample *(Ulva fasciata)*

Microplastics were observed on the sieve and were collected on petri plate using forceps. Visual characterization of microplastics was done based on color, thickness, transparency, hardness, shape. Based on which the isolated microplastics were grouped in different classes with code UL.

Table 5 Visual Characterization of isolated microplastic from Seaweed Sample

designated mp	no. of microplastic particles	color	thickness	transparency	hard/ soft	weight (mg)	Shape
UL01	6	—	thin	transparent	soft	—	fiber like

18 g of seaweed contained 6 microplastic particles.

4.1.3.1 Microscopic analysis and microplastic scaling

Images depicting the microplastics from seaweed sample with scale under the microscope:

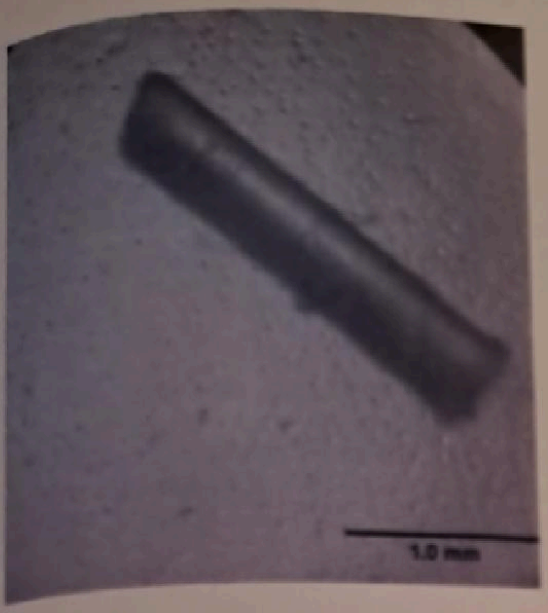

Figure 27: UL01

4.1.3.2. Raman Spectra with Standard plastic polymer spectrum for microplastics in seaweed sample: (All spectrum above 70% similarity were chosen)

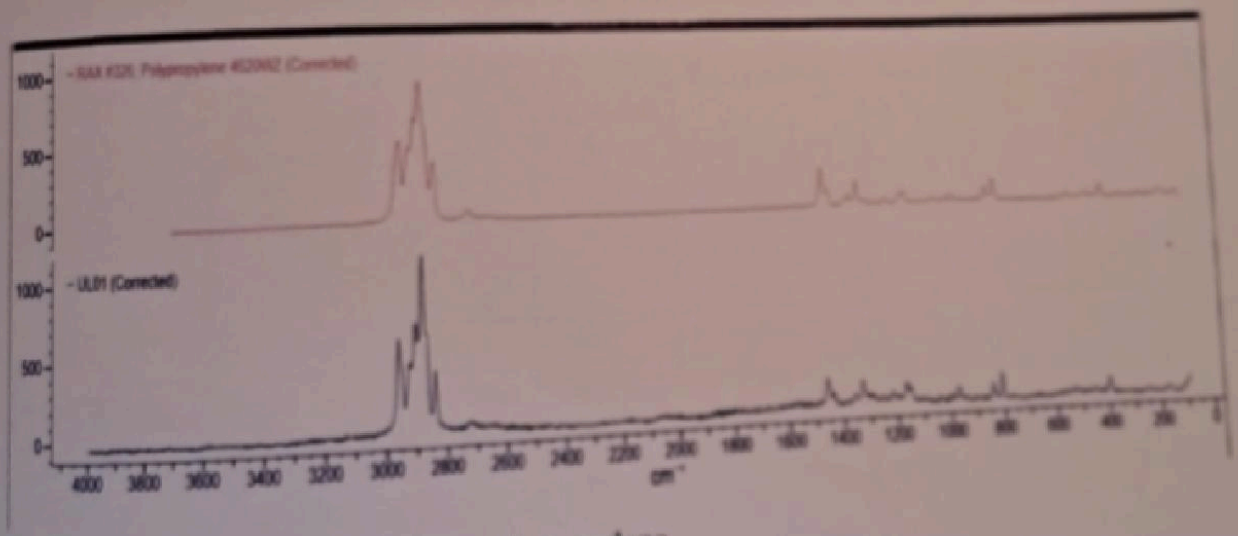

FIGURE 28: Raman spectrum for polypropylene.

Microplastic in class UL01 showed similarity to polypropylene.

From the Seaweed sample, 6 microplastic particles were isolated. After visual characterization of microplastics they were classified into a single class. One microplastic from the class was scaled

using light microscopy with hemocytometer as the reference and was further given for micro-Raman spectroscopy analysis. All the microplastics observed were polypropylene fibers. This result in accordance to the study conducted in Thailand on 2 edible seaweeds reported that samples exhibited 100% of fibrous microplastics and majority of microplastic identified to be polypropylene (Kolomjit et al, 2021)

4.1.4 Green Mussels: (*Perna viridis*)

Microplastics were observed on the sieve and were collected on petri plate using forceps. Visual characterization of microplastics was done based on color, thickness, transparency, hardness, shape. Based on which the isolated microplastics were grouped in different classes with code MS.

Table 6 Visual Characterization of isolated microplastic from *Perna viridis*
Sample:

designated mp	no. of microplastic particles	color	thickness	transparency	hard/ soft	weight (mg)	Shape
MS01	2	blue	thin	opaque	soft	0.1	thread like
MS02	4	green	thin	opaque	soft	0.2	Small fragments
MS03	5	—	thick	transparent	hard	0.3	Film like

4.1.4.1. Microscopic analysis and microplastic scaling

Images depicting the microplastics from mussel sample with scale under the microscope:

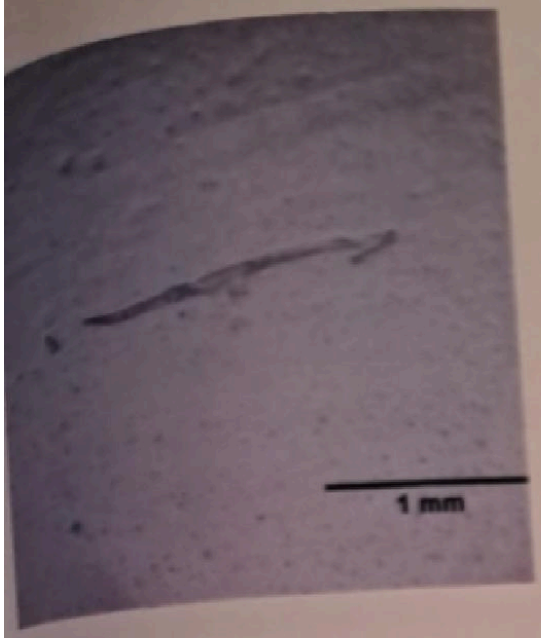

Figure 29: MS01

Figure 30: MS02

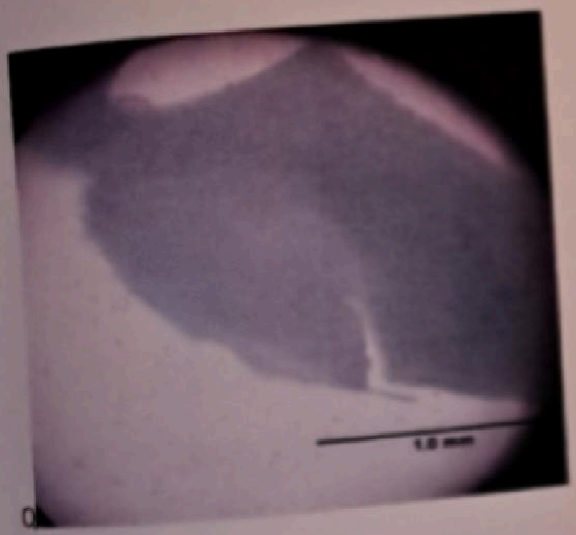

FIGURE 31: MS03

21.5 g of mussels contained 11 microplastic particles.

4.1.4.2 **Raman Spectra with Standard plastic polymer spectrum for microplastics in mussel sample:** (All spectrum above 70% similarity were chosen)

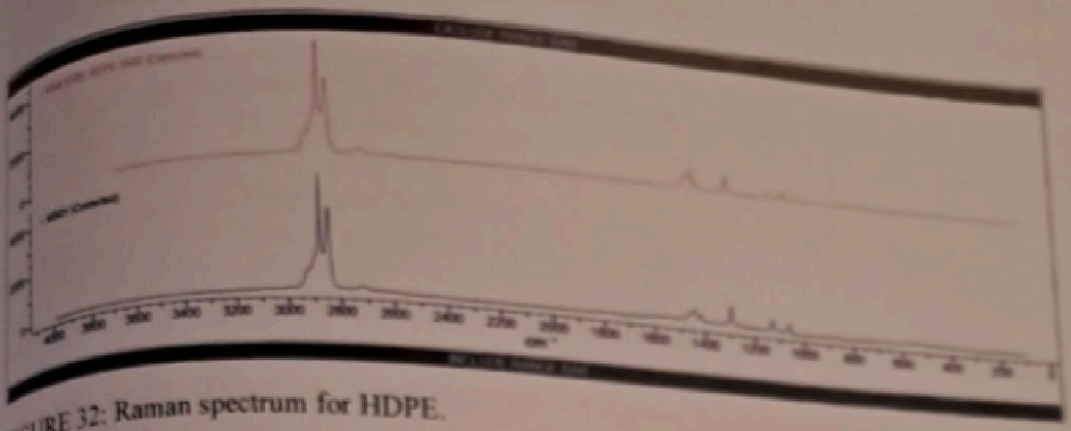

FIGURE 32: Raman spectrum for HDPE.

Microplastic in class MS01 showed similarity to HDPE.

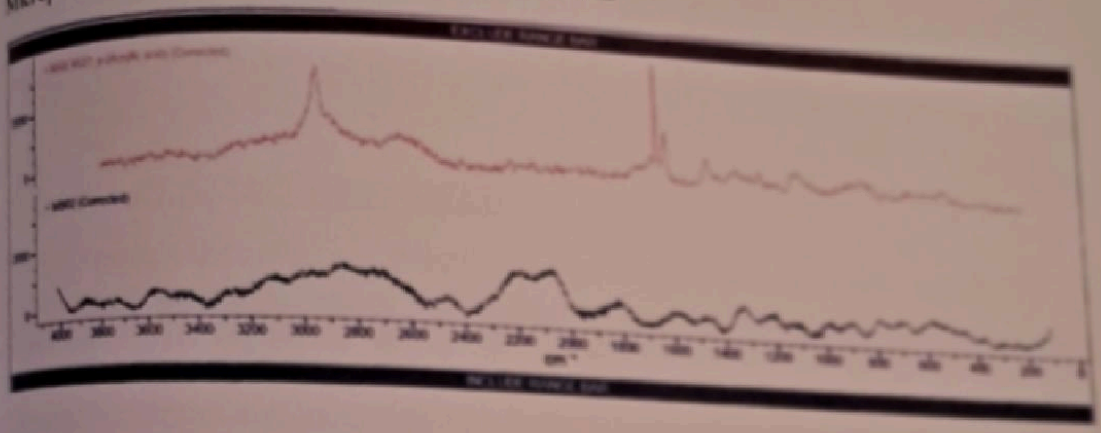

FIGURE 33: Raman spectrum for polyacrylic acid.

Microplastic in class MS02 showed similarity to polyacrylic acid.

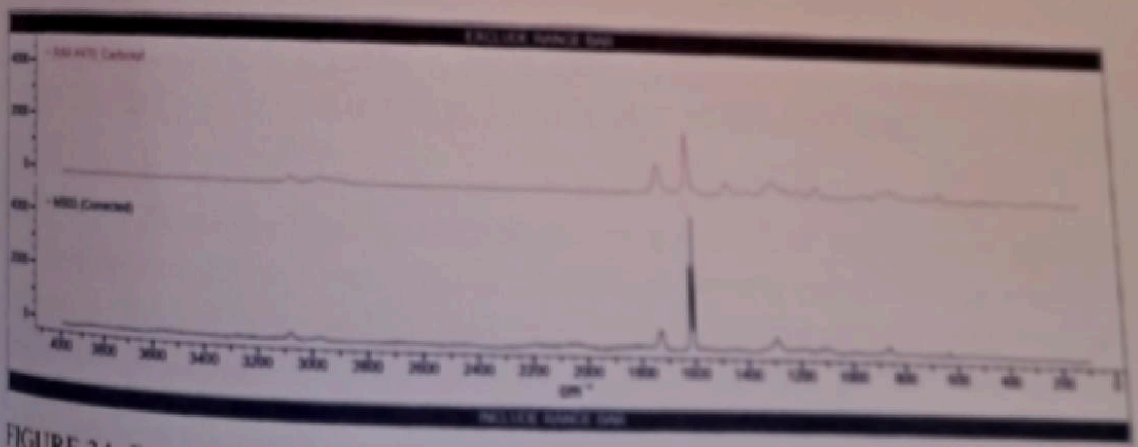

FIGURE 34: Raman spectrum for polyester.

Microplastic in class MS03 showed similarity to polyester.

From 21.5g mussel tissue, 11 microplastic particles were isolated. After visual characterization of microplastics they were classified into 3 classes. One microplastic from each class was scaled using light microscopy with hemocytometer as the reference and was further given for micro-Raman spectroscopy analysis. Most microplastic was in the form of film followed by small fragments and threads. In a similar study on green mussels, fibers, fragments and films were found to be the dominant forms of microplastics observed (Saha et al., 2021). Polyester was found to be the most abundant followed by polyacrylic acid and HDPE (high density polyethylene). In a similar study, Polypropylene, polyethylene, polystyrene and polyethylene terephthalate were detected among microplastics (Leung et al, 2021). Polyacrylic acid is a polyolefin. It can be viewed as polyethylene with carboxylic acid (CO_2H) substituents on alternating carbons. Polyacrylic acid and its derivatives are used in disposable diapers. Acrylic acid is also the main component of Superadsorbent Polymers (SAPs), cross-linked polyacrylates that can absorb and retain more than 100 times of their own weight in liquid.

NANO- PLASTIC ASSESSMENT

For determining the relationship between the Surface polarity of the plastic polymer and the wavelength of the emitted fluorescence when stained with Nile Red.

2 positive controls are taken:

1. PET (Polyethylene terephthalate): Polar
2. PE (Polyethylene): Non- Polar.

PREPERATION OF SAMPLE FOR ANALYZING POLAR PLASTIC POLYMER(PET) UNDER FLOURESCENCE MICROSCOPE.

A piece of PET was cut into smaller fragments and washed with ethanol, followed by washing with filtered water and air dried. This sample is mounted on a slide and stained with a few drops of Nile Red. Incubated for30 min prior to visualization under the microscope.

OBSERVATION-

FIGURE 35: Fluorescence microscopic image of nile red stained PET sample

RESUT- PET gave red fluorescence under blue light when stained with Nile Red.

- PREPERATION OF SAMPLE FOR ANALYZING NON- POLAR PLASTIC POLYMER(PE) UNDER FLOURESCENCE MICROSCOPE.

A piece of PE is cut into smaller fragment and washed with ethanol, followed by washing with filtered water and air dried. This sample is mounted on a slide and stained with few drops of Nile Red. Incubated for 30 min prior to visualization under the microscope.

OBSERVATION-

FIGURE 36: Fluorescence microscopic image of nile red stained PE sample

RESULT- PE gave green fluorescence under blue light when stained with nile red.

Discussion- NR fluorescence emission spectrum red-shifts markedly as the polarity of the polymer surface increases (Maes et al., 2017).

4.2.1 FLUORESCENCE MICROSCOPIC ANALYSIS OF WATER SAMPLE

FIGURE 37

FIGURE 38

FIGURE 39

FIGURE 40

Figure 37-40: Nanoplastic aggregates showing green and red fluorescence under blue light.

17 nanoplastic aggregates were counted in 120µl of filtered surface water sample so itcan be deduced that in 1L sample 1,40,000 nanoplastic aggregates were present.

4.2.2 FLUORESCENCE MICROSCOPIC ANALYSIS FOR SEAWEED SAMPLE:

FIGURE 41

FIGURE 42

FIGURE 43

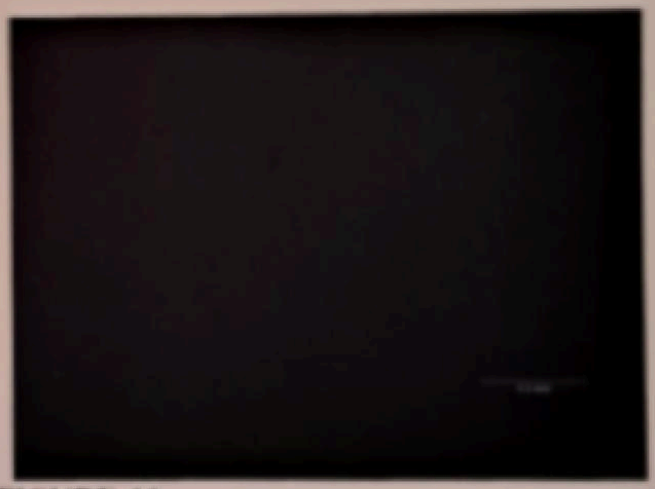

FIGURE 44

Figure 41-44: Nanoplastic aggregates in seaweed sample showing green and red fluorescence under blue light.

74 nanoplastic aggregates were observed on the smeared slides.

2.3 FLUORESCENCE MICROSCOPIC ANALYSIS FOR MUSSEL SAMPLE:

FIGURE 45

FIGURE 46

FIGURE 47

FIGURE 48

FIGURE 49

FIGURE 50

FIGURE 51

FIGURE 52

Figure 45-52: Nanoplastic aggregates in mussel sample showing green and red fluorescence under blue light.

188 nanoplastic aggregates were observed in the smeared slides.

Slightly bigger particles are observed under fluorescence microscope because of the ability of nanoplastics to form homoaggregation and heteroaggregates by binding with clay and other colloidal organic matter. Bhattacharya et al, (2010) measured substantial binding or heteroaggregation of 20 nm polystyrene particles with freshwater phytoplankton cells.

Wegner et al. (2012) were the first to measure and model the homoaggregation of 30 nm polystyreneparticles in seawater and found rapid formation of 1000 nm aggregates within 16 minutes.

CHAPTER 5:

SUMMARY & FUTURE PROSPECTS

5.1. SUMMARY

In this study, assessment of microplastic and nanoplastic in Vagator beach was carried out. Four different samples such as water sample, sediment sample, seaweed sample (Ulva fasciata) and green mussel sample (*Perna viridis*) were assessed. The isolated microplastics were visually characterized based on color, thickness, hardness, transparency and shape. And were classified into classes. A total of 38 microplastics were isolated from the samples and were classified into 11 classes. Mjority of the microplastics were in the form of fragments (14) followed by films (10), threads (8) and fibers (6). And were mostly in the shades of green, white, blue, and transparent.

Raman spectroscopic analysis done by Micro- Raman spectrophotometer and spectrum similarity search done using KnowItAll Information System 2021 by Wiley Online Raman Database.

Identified microplastic polymers as polyethylene, HDPE, polypropylene, poly acrylic acid, diamond like carbon, PET, PBT and polyester. Polypropylene was the major microplastic isolated from surface water sample followed by polyacrylic acid. In sediment sample, PET was found to be the most abundant microplastic followed by PBT. The seaweed sample contained a heavy amount of polypropylene. Polyester is the major microplastic identified in mussel sample followed by polyacrylic acid and HDPE. However, SS02 did not show similarity to any polymer.

Counting and identification of nanoplastic was done by nile red staining technique. According to literature, microplastic and nanoplastic can be classified into polar and hydrophobic based on their polar characteristics as nile red emission spectrum shifts depending on the polarity of its environment. As the polarity increases its fluorescence emission spectrum red shifts markedly. 2 positive controls were chosen to check its efficacy. PET polymer (polar) when stained with nile red showed red fluorescence under blue light in epifluorescence microscope while PE polymer (non-polar) when stained with nile red and observed under same condition gave green fluorescence. Hence, the results were in accordance with the literature. Similarly, the samples were passed through 0.2µm whatman membrane filter and this filtrate was stained with nile red

to check for the presence of nanoplastic. For water sample around 17 nanoplastic particles were observed in 120µl of the filtrate and so it can be deduced that in 1L water sample around 1,40,000 particles of nanoplastic were present. For the seaweed and mussel sample around 74 nanoplastic aggregates and 188 nanoplastic aggregates were observed on smeared slides respectively. It was observed from the fluorescence images that all the three samples, water sample, seaweed sample and mussel sample contained both polar and non- polar plastic polymers however the abundance of non-polar plastic polymers is more, as more green fluorescence was detected. This result is also in accordance with Raman spectroscopic identification for isolated microplastic. As 7 different plastic polymers were identified (Polypropylene, polyethylene, HDPE, Poly acrylic acid, PET, PBT, Polyester) out of which 4polymer are polar (polypropylene, polyethylene, HDPE, Polyacrylic acid) and 3 hydrophobic polymers (PET, PBT and polyester).

5.2 Future Prospects:

- Bioaccumulation of microplastic and nanoplastic in marine ecosystems
- Toxicological studies and the effect of prolonged exposure of various polymers on marine biodiversity.
- Bioprospecting of microplastic and nanoplastic degrading bacteria.

CHAPTER 5:

BIBLIOGRAPHY

5.1 BIBLIOGRAPHY

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CHAPTER 6:
APPENDIX

Reagent preparation 10% KOH

Dissolve 10g of Potassium Hydroxide flakes in 100ml of distilled water.

Preparation of Nile Red Stain:

Nile Red is prepared in acetone to make a working concentration of 1000µg/ml. it should be stored at 4℃ in dark.

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

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

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