Studies on Microorganisms Associated with Plastic Marine Debris

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DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, "Studies on Microorganisms Associated with Plastic Marine Debris" is based on the results of investigations carried out by me in the M.Sc. Environment Science at the School of Earth, Ocean and Atmospheric Sciences, Goa University under the Supervision of Dr. Priya D'Costa 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.

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COMPLETION CERTIFICATE

This is to certify that the dissertation report **"Studies on Microorganisms Associated with Plastic Marine Debris."** Is a bonafide work carried out by Ms Srushti S. Gadekar under my supervision in partial fulfilment of the requirements for the award of the degree of Masters of Science in the Discipline Environmental Science at the School of Earth, Ocean and Atmospheric Sciences, Goa University.

Date: 02/05/2024

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PREFACE

The pristine beaches of Goa, renowned globally for their beauty and allure, harbor a hidden menace beneath their picturesque surface: plastic marine debris. Miramar and Calangute Beach, two of the most frequented tourist destinations in Goa, unfortunately, contribute significantly to the plastic pollution crisis plaguing our oceans. Recognizing the urgent need to address this environmental challenge, this study delves into the intricate relationship between microorganisms and plastic marine debris. Focused primarily on bacterial communities, this research aims to uncover the extent of microbial colonization on plastic debris and, more importantly, identify potential human pathogens associated with these pollutants. By elucidating the microbial dynamics in such environments, we seek to shed light on the implications of plastic pollution not only for marine ecosystems but also for human health. The juxtaposition of tourism and pollution in these coastal paradises provides a unique backdrop for our investigation, highlighting the interconnectedness of human activities and environmental degradation. Through meticulous field sampling and rigorous laboratory analyses, we endeavour to contribute to the growing body of knowledge on the impacts of plastic pollution on microbial ecology and public health.

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LIST OF ABBREVIATIONS

Abbreviation	Full-form
PMD	Plastic Marine Debris
MP	Microplastic
MPs	Microplastic polymers
DNA	Deoxyribonucleic acid
HMs	Heavy metals
ABs	Antibiotics
MRGs	Metal Resistant Genes
ARGs	Antibiotic Resistance Genes
NIS	Non- indigenous species
HAB	Harmful algal bloom
FIO	Faecal indicator organisms
POPs	Persistant organic pollutant
PAHs	Polycyclic aromatic hydrocarbons
PVC	Polyvinyl chloride
ZMA	Zobell Marine Agar
MSA	Mannitol Salt Agar
EMB	Eosin Methylene Blue
TCBS	Thiosulphate- Citrate- Bile Salts Sucrose
℃	Degree celcious

ABSTRACT

Plastic pollution in marine environments poses a significant threat to both ecosystems and human health. This dissertation investigates the presence of microorganisms associated with plastic marine debris at two popular tourist beaches of Goa: Miramar beach and Calangute beach. Five Plastic samples found in the moist soil were collected and affter transportation on ice to the laboratory, they underwent isolation and characterization of plastisphere bacteria.

Five types of media were utilized for bacterial isolation: ZMA for marine bacteria, MSA for *Staphylococcus aureus*, XLD for *Salmonella and Shigella*, TCBS for *Vibrio*, and EMB for *Escherichia coli*. The incubation period was 24 hours, room temperature for ZMA and for rests 24 hours, 37℃. Following an incubation period, subculturing of plastisphere bacterial isolates was performed. A crystal violet assay was conducted to assess bacterial adherence to plastic surfaces using sterile microtitre plates made up of POLYSTYRENE plastic.

Results revealed the presence of human pathogens, including *Vibrio parahaemolyticus* (VPLO) and *Vibrio cholerae* (VCLO) on both beaches, as well as *Salmonella* on Miramar beach plastic samples. These findings raise concerns regarding the potential transmission of harmful diseases via plastic debris, which can serve as a vector for pathogen dispersal over long distances. The implications of these findings extend beyond environmental conservation to include public health considerations, highlighting the urgent need for mitigation strategies to address plastic pollution and its associated risks to both marine ecosystems and human populations.

KEYWORDS

Microorganisms; Plastisphere; Marine; Debris; Miramar; Calangute; Beach

CHAPTER 1: INTRODUCTION

1.1 Background

1.1.1 Plastic pollution in the marine environment

Plastic litter enters the ocean through aquatic and land-based sources, such as rivers and municipal waste treatment plants. Plastic items in the marine environment accumulate at the sea surface due to winds and currents, while negatively buoyant items sink out of the water column. UV radiation exposure of plastic objects on surface waters typically leads to their photodegradation by oxidation. Mechanical, photochemical, and biological forces break down plastic into microplastics (size <5mm) and nanoplastics, which are incorporated into the marine food web (Luna et al., 2017). Public awareness of plastic pollution in our environment has grown as global plastics production, which surpassed 350 million tonnes in 2017, has continued to rise. There is growing public pressure and legislative action to reduce the amount of plastic debris entering our ocean and the environment at large, following the United States and Canada's recent restrictions on the use of microbeads in cosmetics and the European Commission's decision to outlaw some single-use plastics (set to take effect in 2021). Five to thirteen million tonnes of plastic entered the water in 2010, adding to the fifteen to fifty-one trillion floating plastic particles that are present in the marine ecosystem. Investigations on the effects of plastic waste on aquatic environments, including the open and coastal seas, have been the main focus of research. Long-lasting and easily colonized by microorganisms, plastic waste facilitates the establishment of microbial biofilms including potentially pathogenic germs as well as toxic algal bloom species. The term "plastisphere" refers to this newly created biosphere created by humans. About 80% of plastic waste comes from land-based sources, such as rivers and wastewater treatment plants that inadvertently leak microplastics into the environment. It has previously been estimated that the biomass of the known plastisphere makes up between 0.01 and 0.2% of the total microbial biomass in

surface waters of open oceans. Microscopy was the main tool used in early investigations of the plastisphere to identify visually distinct organisms such filamentous bacteria and diatoms.

However, the use of cutting-edge molecular techniques, particularly high-throughput DNA sequencing, is expanding our knowledge of the various microorganisms that live in the plastisphere. Many of the bacteria that live on plastic waste are naturally occurring biofilm formers that favor an attached lifestyle to a free one, according to recent studies that have examined these microbes.

Organisms like filter feeders which can filter large volumes of water, further concentrate these particles. Microorganisms, including potentially harmful pathogens, colonize plastic within hours. The density of plastic affects its lifecycle, with some remaining afloat or sinking into the water column, as they become weighted down by biofouling. The diagram illustrates the various pathways plastic debris takes throughout its lifecycle (Amaral-Zettler et al., 2020)

Upon reaching the ocean, plastic is colonized by various microbes, including archaea, bacteria, and eukarya. Marine viruses may be part of the epiplastic assemblage. These organisms colonizing the plastic debris are collectively called the ‘plastisphere’. The hydrophobic nature of plastic surfaces stimulates the formation of microbial biofilm, which influences the plastic's fate, ballasting ability, degradation, fragmentation, and toxicity level. The transport mechanisms of biofouled microplastics to the ocean interior remain unclear. Once at the seafloor, plastic debris undergoes colonization, degradation, and sequestration.

1.1.2 The plastisphere community.

Conceptual model depicting a microbial ecosystem comprising bacteria, protists, and animals in the oligotrophic open ocean, representing the complex, three-dimensional

plastisphere community. Members comprise primary producers of cyanobacteria and diatoms, predatory ciliates and hydroids, grazers such as ciliates and bryozoans, symbiotic associations, and heterotrophs. Recent research combining molecular data and scanning electron microscopy (SEM) has verified that the plastisphere can be a crowded, surface-based micro-ecosystem of primary producers (like phototrophs), predators, symbionts, and decomposers in the oligotrophic open ocean. A study using SEM and molecular data found a symbiosis between Ephelota and its sulfide-oxidizing bacteria. Plastic debris provides surface attachment for these suctorian ciliates. Co-occurrence networks show positive associations between Amoebophrya and Suessiaceae on polyethylene, despite Amoebophrya typically being parasitic on other dinoflagellates.

1.1.3 Formation of the plastisphere

Microorganisms colonize the surface of microplastic polymers (MPs) sequentially, first overcoming the hydrophobic surface. They can easily colonize within minutes but take time to form a stable biofilm. Lifshitz–van der Waals forces and the electrostatic contact between the substrate and the cell surface function indirectly and reversibly during the early phase of bacterial adherence to the surface, and can result in greater adhesion through adhesion receptor attachment. Later on, Extracellular polymers, LPS, or proteins are generated in an already irreversible stage of adhesion, and the microcolonies grow as a result of the initial adhering cells proliferating (Porkony et al., 2022)

Bacterial cells are connected through a polymeric substance (EPS) that consists of polysaccharides, proteins, nucleic acids, surfactants, lipids, and water. EPS plays a crucial role in biofilm formation and function, providing a protective barrier, avoiding host-immune responses, and facilitating cell aggregation. The biofilm's supply of nutrients is facilitated by the formation of tubules and fluid flowing within it, enveloping microcolonies. However,

most tubules are located at the periphery of microcolonies, preventing access to all cells. This results in partially cut-off bacteria in anabiosis.

Early MP settlers are Gammaproteobacteria and Alphahaproteobacteria, known as pioneer colonizers. Gammaproteobacteria dominate early biofilms on most plastic polymer types. Alteromonas, Thalassobius, Neptuniibacter, Poseobacter, and Phodobacteriaceae also appear in the early stages of biofilm formation. Diatoms and cyanobacteria are also identified at this stage.

Over time, the number of Bacteroidetes, particularly Flavobacteriaceae, increases due to their wide distribution, adaptability, and ability to utilize organic substrates. These secondary colonizers take several months to form, with primary biofilms taking about a week or less to be observed. In the early phase (30 days), the main colonizers were Flavobacteriaceae, Rhodobacteraceae, and Microtrichaceae. In the mid-term (75 days), dominant populations shifted from Flavobacteriaceae and Erythrobacteriaceae to Bacillaceae and Moraxellaceae. At the later stage (135 days), the dominant family shifted back to Flavobacteriaceae, while a significant increase in these bacteria occurred in Rhodobacteraceae, Microtrichaceae, and Piellulaceae. The rate of biofilm formation is likely related to the total nutrients and microorganisms in the marine environment where the plastic is located.

1.1.4 Microplastics as a vector of pathogens

Plastic presence in aquatic environments poses significant threats to both micro- and macro-organisms, leading to intoxication, damage to internal organs, and disturbance of the microbiome. The presence of plastic is closely linked to the affinity of bacteria to its surface, with biofilms easily creating on different types. This presence facilitates ingestion by lower-

linked organisms, such as farmed fish. The durability and mobility of plastic make it a perfect vector for pathogens, potentially affecting organisms kept in closed systems, consumers of aquatic foods, and aquacultural staff (Porkony et al., 2022).

1.1.5 Plastisphere: a potential hotspot for evolution of antimicrobial resistance

The plastisphere provides an environment that encourages microbial adhesion and the subsequent formation of biofilms. Additionally, MP-associated contaminants including heavy metals (HMs) and Antibiotics (Abs) exist in close proximity to the microorganisms in this habitat. Pollution and biofilm can alter microbial species distribution, with ABs being the primary drivers of AMR. ABs are primarily driven by inefficient wastewater treatment processes and pharmaceutical discharge. Heavy metals (HMs) accumulate in the environment through industrial activities and natural processes like bedrock weathering.

HM pollution drives the selection for metal resistance genes (MRGs) and correlates with increased occurrences and amounts of antibiotic resistance genes (ARGs). AMR is promoted in plastispheres through cross-resistance, where resistance mechanisms to HMs and ABs are physiologically coupled, and co-resistance, where antibiotic resistance genes (ARGs) and metal resistance genes (MRGs) are present on the same mobile genetic element, leading to automatic selection of ARGs in metal-resistant environments like animal gut and anthropogenically contaminated soils and water bodies (Kahru et al., 2021).

Thus, in view of the above, the analysis of plastic-borne bacterial pathogens is a matter of great importance, and highlights the presence of one more reservoir in the marine environment (the plastisphere) where bacterial pathogens could occur in high numbers. This will have far-reaching implications on human health.

1.2: Aim and objectives

Aim:

To investigate the presence of microorganisms associated with plastic marine debris, with a focus on bacterial plastisphere communities, as indicators of potential human pathogens, at Miramar Beach and Calangute Beach in Goa.

Objectives:

- To isolate and characterize bacteria associated with plastic marine debris.
- To determine the presence of human pathogens associated with plastic marine debris.

1.3: Hypotheses

- Whether the isolated plastisphere bacteria are indicative of human pathogens.
- Whether the plastisphere bacteria strongly attach to the plastic

1.4: Scope

The presence of pathogens within the plastisphere raises concerns about potential risks to human health and ecosystem integrity. While much of the research on plastisphere microbiology has focused on bacteria involved in plastic degradation and ecosystem processes, there is growing recognition of the presence and persistence of pathogenic bacteria associated with plastic debris in aquatic environments. Pathogens such as *Escherichia coli*, *Vibrio* species, and various opportunistic pathogens have been detected on plastic surfaces, highlighting the potential for transmission to humans through direct contact or ingestion of contaminated water or seafood. Additionally, the ability of bacteria to form biofilms on

plastic surfaces enhances their resilience and may prolong the survival of pathogens in the environment. Furthermore, plastic debris can serve as vectors for the dispersal of pathogens over long distances, exacerbating public health risks. As plastic pollution continues to accumulate in marine and freshwater ecosystems, understanding the scope of pathogenic bacteria within the plastisphere and their implications for human health is essential for developing effective management strategies to mitigate potential risks.

CHAPTER 2: LITERATURE REVIEW

2:1 Plastic pollution and plastisphere

There has been a build-up of plastic debris in the marine environment since the middle of the 20th century, coinciding with an increase in the production of plastic worldwide. Items made of persistant plastic seldom break down, instead, they gradually shatter and are scattered by wind and currents. As a result, marine plastic debris is present in marine environments worldwide (Kirstein et al., 2019).

Physical, chemical, and biological processes break down and weather the plastic that accumulates in the environment, eventually forming microplastics. The environment's microplastic buoyancy and their hydrophobic, organic polymer surface create a special niche for microbes (Zettler et al., 2013).

The Plastisphere is the term for this newly created ecosystem by humans. The word "plastisphere" was first used to describe life on plastic debris (less than 5 mm) that was retrieved from the North Atlantic. Subtropical Gyre, where the floating debris accumulations were referred to as "garbage patches," but the term has subsequently been applied to characterize the life associated with plastic waste in a variety of aquatic locations (Zettler et al., 2013, Amaral-Zettler et al., 2020). Plastisphere a unique microbial community that is associated with plastic and that is not present in the surroundings (Zettler et al.,2013). produced the first significant work that popularized the term "plastisphere' by proving that the microbial populations on plastic pellets in the North Atlantic were different from those in the surrounding saltwater.

2.2: Plastics as vectors for pathogens and non-invasive species

Research findings have consistently indicated the existence of many diseases and detrimental algal bloom species inside the plastisphere (Kirstein et al., 2019). In addition to posing a serious danger to ecosystem services, human health and biodiversity, the introduction and spread of marine non-indigenous species (NIS) and viruses into new environments can have significant negative economic effects.

The primary vector for marine biological invasions is thought to be shipping, the increasing spread of diseases and marine NIS rafts on marine plastic debris (MPD) is less fully understood. Plastics, found in sediments and beach sands, pose a potential reservoir of FIOs and pathogens. This raises research questions about plastics as a vector for public health risks. Floating plastic debris in bathing waters could also contribute to negative health impacts on bathers and recreational water users due to the underexplored potential of plastic litter to harbor and transmit diseases. As a result, plastic trash acts as a substrate that can facilitate the spread of many different organisms, such as potentially harmful *Vibrio* species, barnacles, and bryozoans, as well as hazardous algal species. The increase in stranded and drifting plastic debris along beaches and coastal areas is predicted due to projected sea level, wind speed, wave height, and altered rainfall conditions (Kirstein et al., 2019).

2.3: Plastisphere community

The first investigation on the composition of the plastisphere community utilizing NGS technique was carried out by Zettler et al. (2013). The composition of the plastisphere community differs from that of the surround environment (Amaral-Zettler et al.,2015; De Tender et al.,2015). The surface plastisphere is said to be dominated by photoautotrophic bacteria such as cyanobacteria (Zettler et al., 2013; De Tender et al., 2017; Dussud et al.,

2018), while Bacteroidetes and Proteobacteria are predominant on the seafloor and subsurface plastisphere (Zettler et al.,2013; Dussud et al., 2018). Furthermore, the season, location, kind of polymer, surface roughness, hydrophobic surface, and size of plastic material all affect the taxonomic content of plastisphere (De Tender et al.,2015).

Unlike natural marine substrates, plastic is more conducive to microbial colonization and biofilm formation due to its hydrophobic surface and longer half-life. Future studies should look at the microbial deterioration of plastics and plastic as a metabolic substrate. Research on the possibility of a "core" plastisphere community dedicated to plastic waste is still under progress (Amaral-Zettler et al., 2020).

2.4: The use of plastic as a rafting material, the development of biofilms, and the possibility of hazardous microbes being transported

In addition to the natural mechanisms promoted by materials like rafts of plant, timber, or pumice, plastic trash can offer a new avenue for the spread of alien and invasive species (Goldstein et al., 2014). Macroplastics are already home to a wide variety of organisms, which has occasionally resulted in the introduction of non-native species into previously uninhabited areas. The idea that plastic can offer microbes a novel way to move across marine and coastal ecosystems both spatially and temporally has, however, received very little attention until recently (Amaral-Zettler et al., 2015). The physical characteristics of plastic can offer a special environment suitable for a variety of microbial populations (Zettler et al., 2013). Plastic's buoyant and enduring qualities may help the microbial hitchhikers that attach themselves to its surface survive and travel over great distances. The biofilms that occupy this so-called plastisphere may also serve as habitat for harmful algal bloom (HAB) species, faecal indicator organisms (FIOs), and pathogenic bacteria. Thus, plastic waste may

be serving as a possible vector for the widespread spread of these microbes (Zettler et al., 2013).

2.5: Dispersal of dangerous and infectious microorganisms through plastics

Another process that facilitates the migration of non-native organisms in marine environment is an increase in anthropogenic trash, particularly plastic litter (Bravo et al., 2011). Since plastic is not biodegradable, it lasts longer in the marine environment, which greatly increases the possibility that invasive and alien species would spread widely. Plastic has been demonstrated to have a higher species diversity than other floating substrates, though this is likely to depend on the location and experimental sampling duration. Plastic's buoyancy and durability make it an attractive alternative substratum for a range of colonizers. (Bravo et al., 2011).

Native benthic invertebrates that attach themselves to marine plastic waste have been shown to have increased survivability and long-distance movement. According to one study, diseases were brought into a coral reef ecosystem via drifting plastic debris (Goldstein et al., 2014). Because of its strength and low weight, plastic is a good medium for the spread of non-native organisms. For instance, a piece of flotsam was recently found in the Netherlands that included remnants of tropical biota, including self-fertilizing corals. As per Goldstein et al. (2013), the most of plastic debris items in the North Pacific are minute pieces; nevertheless, this study discovered that these particles only carry a limited of species. of which, like Jellyella or *Membranipora* bryozoans, are well-known subtropical rafters. observed that most displaced taxa were located on large objects, including net balls, but that the coral pathogen *Halofolliculina* spp. was located on medium-sized plastic fragments (0.03–0.1 m^2).

2.6: Impact of plastic pollution and plastisphere on marine ecosystems

Factors such as plastic type, location, and environmental conditions shape the composition and diversity of plastics that have become pervasive pollutants in marine environments, with approximately 8 million tons entering oceans annually. The plastisphere, a microbial community colonizing plastic surfaces, has gained attention due to its potential ecological ramifications. This review synthesizes current knowledge regarding the influence of plastisphere on marine ecosystems. The plastisphere hosts diverse microbial communities, including bacteria, archaea, fungi, and protists (Zettler et al., 2013). These organisms adhere to plastisphere communities. Plastisphere interacts with marine organisms across various trophic levels, from plankton to apex predators. Research indicates that plastic debris serves as vectors for the transport of invasive species. Additionally, ingestion of plastic particles by marine animals can lead to physical harm, digestive tract blockage, and bioaccumulation of toxic substances.

Plastisphere alters marine ecosystems through multiple pathways. The presence of plastic debris disrupts nutrient cycling, habitat structure, and species interactions (Dussud et al., 2018). Moreover, microbial activity on plastic surfaces may accelerate the degradation of organic matter, impacting carbon fluxes and ecosystem productivity (Kirstein et al., 2016). Plastics adsorb and concentrate organic pollutants from seawater, creating contamination hotspots in plastisphere biofilm. These pollutants include persistent organic pollutants (POPs), heavy metals, and microplastics-associated chemicals. Marine organisms interacting with plastisphere may face heightened exposure to these contaminants, posing risks to their health and fitness. Addressing plastisphere's impact on marine ecosystems necessitates integrated management approaches. Efforts to reduce plastic pollution, such as enhanced waste management and recycling initiatives, are crucial (Galgani et al., 2013). Additionally, research into innovative technologies for plastic cleanup and biodegradation can help

mitigate plastisphere's environmental consequences. The plastisphere represents a complex interface between plastics and marine ecosystems, with profound implications for biodiversity, ecosystem function, and human well-being. Understanding plastisphere dynamics and its impact on marine environments is essential for developing effective conservation strategies and safeguarding ocean health.

2.7: Harm of plastisphere in recreational activities

The presence of microplastics and plastic leachates in aquatic ecosystems, known as the plastisphere, poses a significant threat to phytoplankton and the overall health of these ecosystem. This is particularly concerning for recreational activities such as swimming and sport fishing, which involve contact with water and can potentially expose individuals to these harmful substances (Keswani et al., 2016). Beaches and coastal environments are crucial ecological and socio-economic habitats worldwide, but they are under significant pressure from anthropogenic activities In Europe, the EU Bathing Water Directive governs the quality and safety of bathing water and beaches. The directive sets standards for microbial water quality and requires the production of a Bathing Water Profile for all designated waters. Designations like the Blue Flag award are also largely driven by the BWD.

Additionally, recreational activities can directly impact natural dunal phytodiversity, with trampling and eutrophication being key factors (Grunewald 2006). These findings highlight the potential harm of the plastisphere in recreational activities, both in terms of direct exposure to harmful substances and the indirect impact on natural ecosystems. The review explores the potential of marine plastics as a mechanism for the persistence and transmission of harmful microorganisms in coastal environments, highlighting the need for sustainable beach management options and understanding the risks of human exposure to plastic pollution.

2.8: Marine debris in food web

Marine debris enters the food web through both direct and indirect means, affecting all trophic levels. When a predator consumes an organism that has either retained the plastic (trophic transfer), the plastic has attached itself to the organism's external surfaces or gills (including entanglement), or the organism has attached itself to the plastic's surface to form a plastisphere, making the plastic an enticing food source, then indirect consumption pathways take place (Savoca et al., 2017).

One of the biotic processes influencing the vertical distributions of plastics in the marine environment is the production of this plastisphere or aggregate. Microbes and phytoplankton attach themselves to the surfaces of plastics to form the plastisphere or aggregate (Zettler et al., 2013). Hydrolysis of the hydrocarbons from the plastic polymers can be aided by the attachment of these bacteria.

2.9: Overcoming the challenges to the plastisphere characterization research gaps.

The most effective method for quantifying and characterizing smaller fractions of MPs, including nanoparticles (<1 μm), is to merge new analytical methods with existing technologies. Combining AFM with infrared spectroscopy or Raman is promising, but quality imaging depends on sample flatness. For a comprehensive overview, Pyrolysis-Gas Chromatography Time of Flight Mass Spectrometry (Py-GCToF) is recommended for aqueous samples. Flow cytometry in combination with staining and cell sorting is another option (Kaile et al., 2020). Merging analytical and sequencing technology with in situ and ex situ experiments can uncover changes in MPs and NPs composition and their affinity for pollutants and microbes. *Ex situ* batch sorption experiments focus on specific parameters, while in situ studies observe complex interactions. Analytical techniques like Py-GCToF, micro-FTIR, Raman spectroscopy, HPLC, and ICP-MS can be used for detection and

characterization. Microbial analysis should include CLSM and metagenomic sequencing (Li et al., 2018).

The accumulation of microplastics (MPs) in the food chain and their impact on the spread of AMR should be studied using long-term in vivo studies and multidisciplinary tools like NGS sequencing, ICP-MS, and vibrational spectroscopy. Previous studies have been inconsistent and yield conflicting results. Standardizing in vivo studies is challenging due to sample material complexity. An enzymatic purification method developed can solve this issue by removing organic and inorganic material from different matrices without affecting polymers. This method can be coupled to micro-FTIR and Raman spectroscopy (Li et al., 2018).

2.10: Plastic degradation process

Biodegradable bags degrade significantly faster than polyethylene bags, with 100% degradation occurring between 16 and 24 weeks. Biofilm formation on the plastic bag surface occurs after 15 days of exposure to the marine environment. The amount of biofilm increases significantly within 33 days on both types of bags. The early biofilm formation and composition are influenced by the plastic type and habitat. Mechanical tests did not show a reduction in tensile properties within a month of exposure. However, scanning electron microscopy analysis revealed surface alterations in biodegradable plastic.

The study by Lobelle and Cunliffe (2011) found that biofilm formation can be visible within a week, and the hydrophobic features of polyethylene plastic food bags submerged at the sea-end of Queen Anne's Battery (UK) changed during a 3-week experiment. However, they did not observe polyethylene-degrading organisms. The study also showed that

removing visible biofilm from plastic reverses its physicochemical properties. A recent study by Oberbeckmann et al. (2017) investigated biofilm formation on plastics and found that microplastics were covered by assemblages after 2 weeks of incubation in cold marine water.

Erythrobacter bacteria, known for their ability to utilize polycyclic aromatic hydrocarbons (PAHs), may be capable of degrading PAHs associated with plastic. De Tender et al. (2015) investigated biofilm development on a plastic surface and suggested that modeling biofilms could help identify species involved in biodegradation, although knowledge about microorganisms degrading plastic in cold habitats is limited.

2.11: Interaction between microorganisms and plastic in cold marine habitats

Microorganisms thrive in cold environments like lakes, sea ice, snow, permafrost soils, cloud droplets, rock environments, and glacial ice (Bryant et al., 2016). In marine ecosystems, hundreds of millions of bacteria can be found in a gram of wet marine sediment. Even plastic waste, which can be colonized by microorganisms, is not immune to their influence. However, few studies have explored the interaction between plastic and marine microbiota (Bryant et al., 2016).

The mechanisms of bacterial attachment on plastic surfaces are poorly understood, but they are a strategy for bacteria to survive marine conditions. Bacteria colonize plastic materials quickly, forming microbial assemblages and covering the surface. These assemblages may catalyze metabolic reactions, leading to the adsorption, desorption, and fragmentation of microplastic-associated compounds.

Biofilms are formed by the settlement of microalgae and microscopic fungi, which can vary in abundance. They are referred to as microbial assemblages, biofouling communities, or periphyton, and are also known as the plastisphere. Biofouling increases the density of the particles, potentially sinking them to the seafloor. Biofouled materials may

attract invertebrates that can graze on plastic inhabitants, increasing the biofouling ratio. This process is crucial for maintaining the health of marine ecosystems. Biofouled material transmission leads to the transfer of non-native or invasive species into new marine habitats, potentially affecting marine ecosystems. The impact of this transfer on original ecosystems is still unknown, but it is crucial to investigate the precise relationships between plastic waste and cold-marine habitats to better understand the effects of this transfer (Bryant et al., 2016).

2.12: Potential impacts of plastic pollution and the plastisphere community on ecosystem function and health

The study found a significant functional differentiation between microbiota in the plastisphere and aquatic environments, suggesting microplastic pollution could impact ecosystem functions. Analysis using FAPROTAX and FUNGuild revealed this, but further research with metagenomic and metatranscriptomic technologies is needed (Zettler et al., 2013).

The study reveals that the biogeochemical cycling of elements differs significantly between the plastisphere and aquatic environment, including carbon, nitrogen, and sulfur cycling. Microplastics may affect ecosystems by forming unique microbial communities in the plastisphere, and altering microbial communities in environmental media where pollution occurs. This suggests that microplastics could also impact the elemental cycling function of ecosystems (Li et al., 2020). The plastisphere is rich in chemicals released by microplastics and absorbed from the environment, making it enriched with microorganisms with compound degradation functions. The plastisphere is also enriched with potential pathogenic microorganisms, which pose a significant threat to ecosystem biosecurity and human water security. Microplastics, as long-distance transport carriers, are responsible for these

pathogenic microorganisms (Zettler et al., 2013). The study revealed that the microbiota in the plastisphere and aquatic environment exhibit distinct functional differences in freshwater and seawater ecosystems, suggesting that the plastisphere's ecological impacts vary across different ecosystems, a finding not previously explored (Zettler et al., 2013).

2.13: Different roles of the plastisphere on microbial network in freshwater and seawater ecosystem

The study compared the interaction networks and keystone species of microbiota in the plastisphere and aquatic environments in different ecosystems. Freshwater ecosystems had a less complex network with more modules and competitive links, while seawater ecosystems had the opposite. Modules may be caused by niche differentiation, habitat heterogeneity, and divergent selection. The heterogeneity, disconnection, and fragmentation of the plastisphere as a habitat could increase.

The heterogeneity and disconnection of the plastisphere habitat can increase dispersal limitation, reduce microbial interactions, and promote niche differentiation, reducing network complexity and increasing modularity. The filtering effect of the plastisphere can increase niche overlap of microorganisms on plastic debris, increasing competition between species. However, in seawater ecosystems, high salinity and low nutrient concentrations reduce the biomass and activity of microorganisms, reducing network complexity (Chen et al., 2016)

The resource-ratio hypothesis suggests that species at low resource levels can experience spatial variability, facilitating resource partitioning and niche differentiation, increasing the number of modules and modularity. The harsh environment in seawater may screen microorganisms for salt and barrenness tolerance, increasing the proportion of species with similar niches and intensifying competitive relationships. The plastisphere, which provides

shelter and absorbs organic substances, also plays a role in the harsh environment of seawater ecosystems, providing nutrients for microorganisms (Mincer et al., 2016)

The study suggests that heterogeneity, fragmentation, environmental harshness, and resource deficiencies in habitats can reduce species interactions, decrease network complexity, increase competition links, and promote niche differentiation. The plastisphere plays a different role in freshwater and seawater ecosystems, leading to different microbial functions. Further exploration of the plastisphere's effects in different ecosystems is recommended.

2.14: Cyanobacteria on plastic surfaces

It was discovered that the cyanobacteria colonizing the plastic surface employed a totally different light-harvesting mechanism than the ones in the surrounding seawater, with the former exhibiting a higher expression of genes encoding Chlorophyll a/b-binding light-harvesting protein and the latter using an increased abundance of phycobilisome antenna encoding genes. The presence of cyanobacteria that use phycobilisomes on plastics, along with higher oxygen production and respiration rates on plastics than in background seawater, indicates that plastic particle communities are not experiencing as severe nutrient limitation in the NPSG. According to this, cyanobacteria on plastic surfaces photosynthesize through phycobilisome complexes, whereas cyanobacterial rial photosynthesis in seawater primarily occurs in the chlorophyll-binding complexes. Chlorophyll a/b proteins require fewer amino acids to bind the chromophore than phycobilisome proteins do, and as a result, less nitrogen is needed to synthesize Chl a/b proteins. On plastic surfaces where there are already insufficient nitrogen sources, the high expression of the phycobilisome gene appears to be highly damaging to *cyanobacteria's* ability to survive. Phycobilisome proteins have the benefit of being easily broken down in the absence of nitrogen and having the ability to

swiftly rearrange once a suitable source of nitrogen becomes available. *Phycobilisomes* not only function as light-harvesting complexes but also as nitrogen reservoirs, which boost cyanobacteria survival and offer important benefits in MP surface nitrogen-limited situations. Additionally, microbial communities on the surface of MPs were discovered to have high expression levels of the nitrogen fixing enzymes (Amaral-Zettler et al., 2020)

2.15: Biofouling of plastisphere

Biofouling increases the density of plastic particles, potentially sinking them to the seafloor. This can attract invertebrates, increasing the biofouling ratio. This can lead to the transport of non-native or invasive species, potentially affecting marine ecosystems. Six plastic/rubber materials in marine debris and beach litter were studied in Biscayne Bay, Florida, to determine fouling's effect on buoyancy. Results showed most plastics undergo fouling, causing negative buoyancy and rapid defouling. This study reveals that floating plastic debris samples undergo rapid fouling under exposure conditions, but once submerged, they defouled and may resurface. Fouling also occurs under submerged conditions, producing a different foulant colony (Amaral-Zettler et al., 2020).

The findings suggest that the process of fouling marine plastic debris may be more complex, with longer exposure durations for larger marine animals. Invertebrates that can feed on plastic residents are predicted to be drawn to biofouled materials. A study using scanning electron microscopy analyzed to biodiversity of organisms on the surface of 68 small floating plastics from Australia-wide coastal and oceanic samples. The most diverse group of plastic colonizers was diatoms, with 14 genera. Other organisms included coccolithophores, bryozoans, barnacles, a dinoflagellate, an isopod, a marine worm, marine insect eggs, and rounded, elongated, and spiral cells identified as bacteria, cyanobacteria, and

fungi. The study also observed plastic surface microtextures, suggesting biota may play a role in plastic degradation. The ecological implications of this phenomenon for marine organism dispersal, ocean productivity, and biotransfer of plastic-associated pollutants remain to be elucidated (Dussud et al., 2018). Transport of non-native or alien organisms is a result of the transfer of biofouled material. The presence of microorganisms in one area of marine trash that are naturally present in other areas may have an adverse effect on marine ecosystems.

2.16: Human pathogens

Research shows human pathogens on plastic particles in aquacultural farms pose health and safety risks to consumers and producers. Exposure to bacteria from undercooked seafood and handling sharp-finned fish can cause tissue disruption. *V. cholerae* is the most well-known human pathogenic bacterium, responsible for numerous global epidemics. It has been found on cruise ship ballast waters and plastic fragments, along with *V. mimicus* and *V. vulnificus,* which cause gastroenteritis. *Vibrio* spp., along with *Escherichia coli,* pose significant health risks to consumers. They can infect wounds through contact with contaminated water or *vibrio*-vectoring shellfish (Porkony et al., 2022). *V. parahaemolyticus, V. fluvialis,* and *V. alginolyticus* are known to cause high temperature and severe diarrhea, with the latter being particularly prevalent in Thailand. *Vibrio fluvialis* is an emerging food-borne pathogen, causing diarrheal outbreaks and sporadic extraintestinal cases.

E. coli and *Enterococci* in Brazil's plastisphere, while experimental research in Argentina and China found fecal contamination in MP biofilms, while *E. coli* and *Enterococci* were also found in Turkish dam lakes. Norway has discovered *Moragnella morganii,* a Gram-negative bacillus causing skin and soft tissue infections, pyelonephritis, female genitalia infections, pneumonia, gangrenous appendicitis, and tonsilliti. *Arcobacter,* a genus of pathogens, was found in microplastics from the Humber Estuary, UK, and

Aeromonas veronii, a wound-infecting bacteria, was detected on aquaculture equipment in China.

An experiment comparing the bacterial fauna of polyvinyl chloride (PVC) microplastic pellets and leaves and quartz particles in water sampled from the Haihe River, China. (Razos et al., 2020). They found two opportunistic human pathogens on MPs: *Pseudomonas monteilii* and *P. mendocina*, which cause exacerbation of bronchiectasis and are known to cause various infections. *Pseudomonas* spp. were also found in the estuary of the Yangtze River, aquacultural waters, and equipment in China and Norway. *Shewanella* sp. was found in the Haihe River Estuary in 2019 and may cause infections with or without bacteremia.

CHAPTER 3: METHODOLOGY

3.1: Study Areas

The 2 study sites selected were Miramar beach and Calangute beach, coastal ecosystems of Goa to carry out studies on microorganisms associated with Plastic Marine Debris. The 1st study area was Miramar beach (Figs. 3.1-3.2). It lies between the coordinates 15.48'N and 73.80'E. Tourists are drawn to Miramar Beach by its breathtaking beauty, which is only 3 kilometers away from Goa's capital, Panaji. Miramar beach is located at the estuary of river Mandovi. The Arabian Sea and Mandovi River meet at this beach, which is also referred to as the Gaspar Dias. The Dona Paula Beach is about a kilometer away, so it's really near. One of the most visited beaches in Goa is Miramar. The Portuguese term from which the word "miramar" is derived means "viewing the sea". The palm trees and golden smooth sand of the beach are its most well-known features. It's said that the moonlight makes beach's sand appear more radiant. Both locals and visitors from other countries frequent the well-liked two-kilometer beach. It's a popular spot for strolls down when the sets far below the horizon and visitors are forewarned that crowds often form here in the evenings when locals wander by. Because there are so many entertainment site options, one may frequently observe a mixed audience of visitors and residents here.

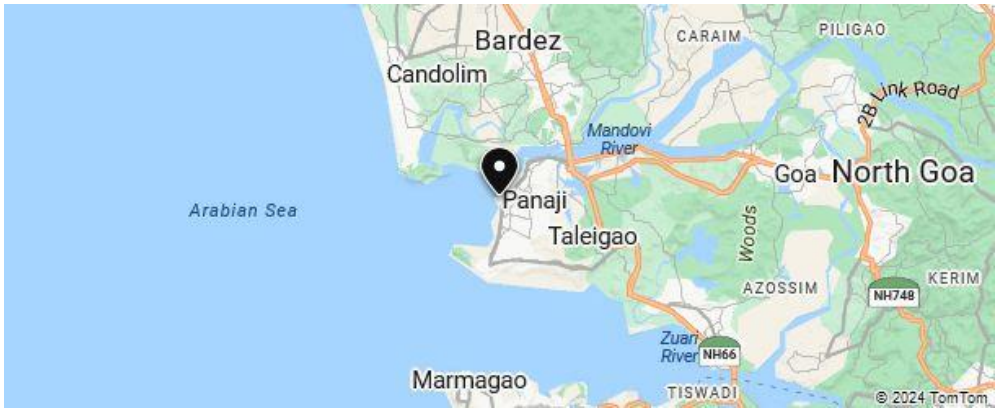

Figure 3.1: Geographical location of 1st sampling site, Miramar Beach.

Figure 3.2: Miramar beach.

The second study site was Calangute beach, a coastal ecosystem of Goa (Figs. 3.3-3.4). It lies between the coordinates 15.54’N and 73.75’E. The longest beach in North Goa, Calangute Beach stretches from Candolim to Baga and is located 15 kilometers from Panajim. It serves as a center for travelers and backcountry enthusiasts from all over the world because of its immense size and appeal. Calangute Beach, located in Goa, is regarded as the "Queen of Beaches" and is ranked in the top ten beaches worldwide for swimming. This is one of Goa's busiest and most commercial beaches. There are several clubs that open up onto the beach, and the beach scattered with shacks. Water sports including parasailing, water surfing, banana rides, and jet skiing are also popular at Calangute Beach. Travelers and backpackers from all over the world are drawn to Calangute Beach, which is crowded with visitors year-round.

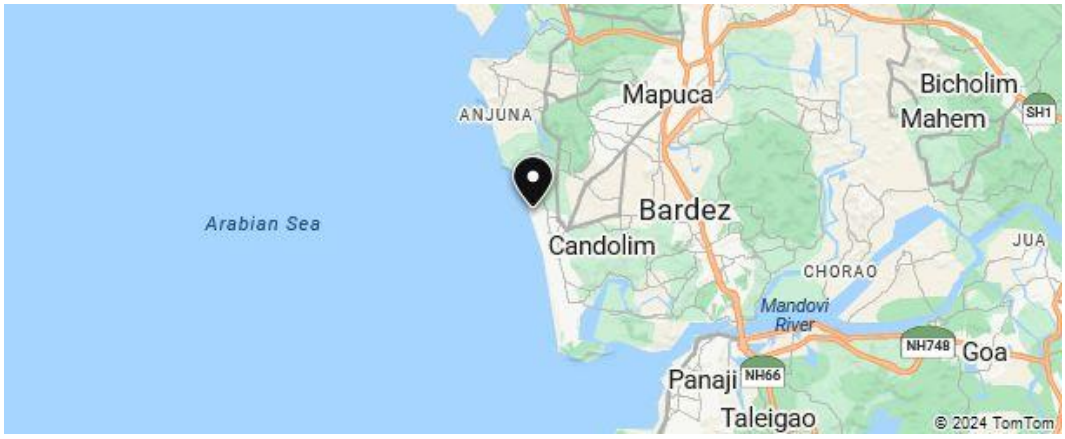

Figure 3.3: Geographical location of second sampling site, Calangute beach.

Figure 3.4: Calangute beach.

Figure 3.5: Plastic pollution in Miramar beach

Many of the plastic samples were embedded in the sand in Miramar (Fig. 3.5). Comparatively, Calangute beach was cleaner with fewer plastic sample observed (Fig. 3.6).

Figure 3.6: Plastic sample embedded in sand in Calangute beach.

Both these study areas are polluted with plastics. Different types of plastics were present on both beaches. Plastics like milk packages, plastic bags, polythene were present in huge number on Miramar beach. Where else chips packets, plastic bags were present on Calangute beach.

3.2: Preparation in the laboratory prior to sampling

3.2.1: Sterilization of seawater to wash plastic samples

Around 2 liters of seawater were collected from the sampling sites in bottles and brought back to the laboratory to filter the water through a filtration unit. 0.2µl (Fig. 3.7). Whatman filter paper was used to filter the water. This filter paper was used in order to trap dirt and unwanted microbes from the seawater. Then this filtered water was put in 500ml bottles and was autoclaved and used for further washing of plastic samples.

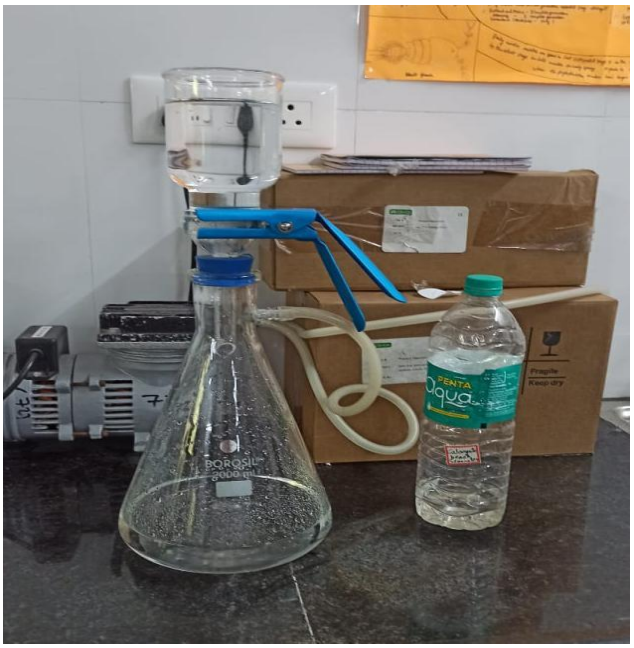

Figure 3.7: Filtration of seawater

3.2.2: Preparation of media

To isolate plastisphere cultures, 5 different media for different groups of bacteria (Table 3.1) were prepared, autoclaved and poured in sterile petriplates. Also, 2 controls were prepared for each media respectively.

Table 3.1: Details of media prepared for specific groups of microorganisms.

Media	**Microorganisms**
Zobell Marine Agar (ZMA)	Marine bacteria
Mannitol Salt Agar (MSA)	*Staphylococcus aureus*
Eosin Methylene Blue (EMB) Agar	*Escherichia coli*
Xylose- Lysine Deoxycholate (XLD) Agar	*Salmonella* and *Shigella*
Thiosulphate-Citrate-Bile Salts Sucrose (TCBS) Agar	*Vibrio* species

3.3: Sampling details:-

First sampling site: Miramar beach

On 17 January 2024, around 10am, sampling of plastics was carried out. Total 5 plastic samples were collected from the sampling site (Fig. 3.8) which were present in the moist soil using sterile forceps and were washed with sterile seawater. Then a piece of plastic was cut using sterilized scissors having size around 20cm x 20cm and was wrapped in aluminium foil and brought back to the laboratory in ice and used for the isolation, identification and characterization of plastisphere bacterial isolates.

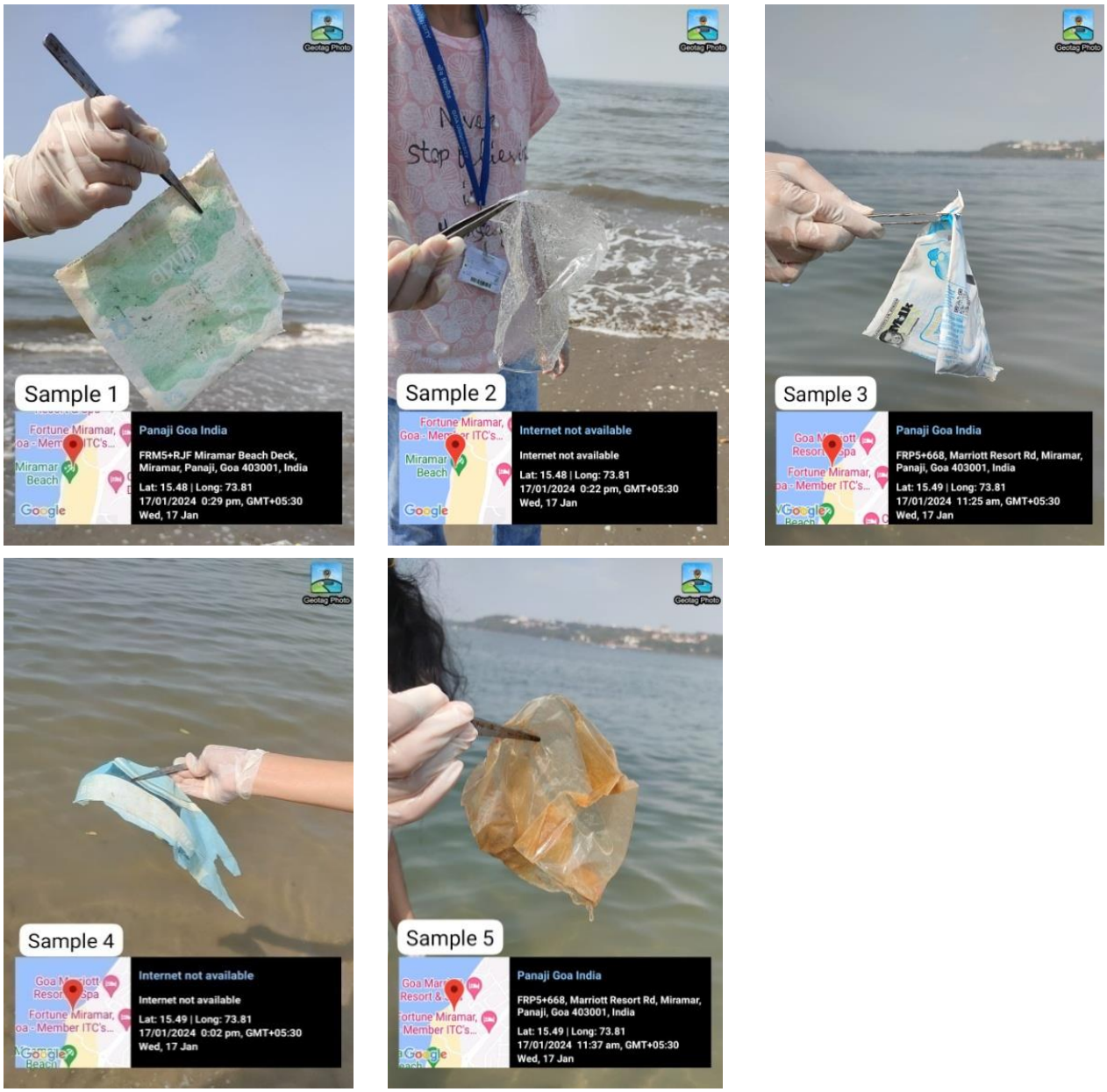

Figure 3.8: Details of samples collected from Miramar beach.

Second sampling site: Calangute beach

On 6 February 2024, around 10am, sampling of plastic was carried out. The same procedure of plastic collection from Miramar beach was followed. Sample details are mentioned in Fig. 3.9.

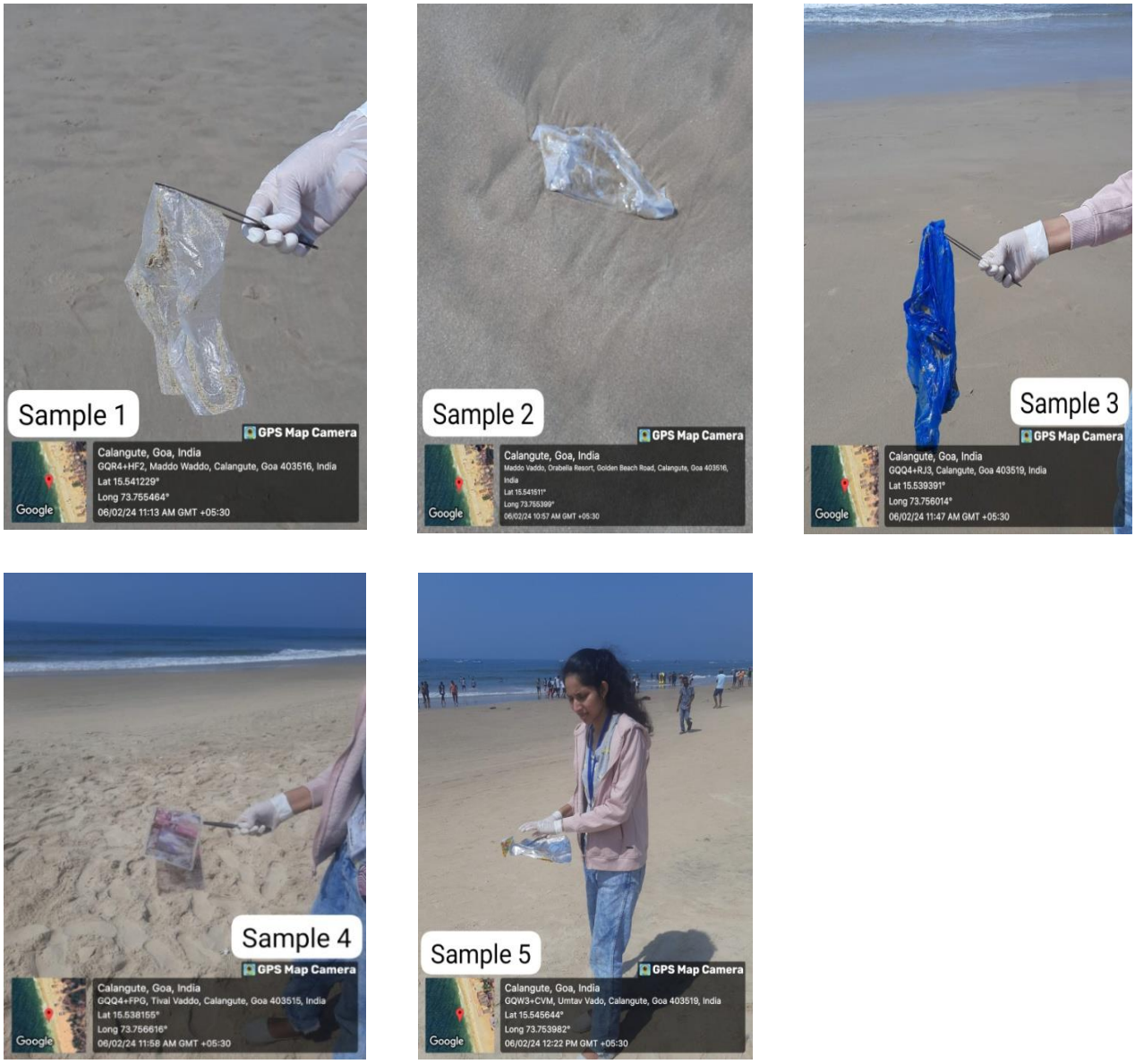

Figure 3.9: Sample collection from Calangute beach.

3.4 Physicochemical parameters

- Temperature:

To check temperature of the water on sampling site, the thermometer was dipped into the surface water. Reading (temperature) was noted down in (degree celsius).

- Salinity

Surface water sample was collected in a centrifuge tube. Using a dropper, water was taken out and 2-3 drops were put onto the refractometer. The lid was closed ensuring no air bubbles were trapped in and viewed through the eyepiece and then salinity reading was noted.

- pH

pH paper was dipped in water sample collected in the centrifuge tube. The colour of the pH paper was observed and pH was checked with the help of pH strip.

3.5: Laboratory analyses

The collected samples were brought back in laboratory in ice. The plastic samples were cut into small pieces and each plastic sample was washed using sterilized sea water in sterile 6 multi-well plates. Each plastic sample was kept on respective 5 media- Zobell Marine Agar (ZMA) for marine bacteria, Xylose-Lysine Deoxycholate (XLD) Agar for *Salmonella* and *Shigella*, Mannitol Salt Agar (MSA) for *Staphylococcus aureus*, Eosin Methylene Blue (EMB) for *Escherichia coli*, Thiosulphate-Citrate-Bile Salts Sucrose (TCBS) Agar for *Vibrio*. The incubation period was 24 hours at room temperature for ZMA and for rest plates 24 hours at 37℃. Also 2 controls were kept, one was closed and one was opened for 10 seconds for each respective media. Then inoculated culture from the plastic samples were picked using sterile loop and quadrant streaking was done on ZMA media in order to obtain pure cultures. Then the pure culture was inoculated on slants containing ZMA media.

3.6.1: Isolation of plastisphere bacteria

The colonies obtained after incubation were purified by following the streak plate method.

The streak plate method is a technique used in microbiology to isolate and purify bacterial colonies from a mixed culture. The method involves using a sterile inoculating loop to streak a small amount of the mixed culture onto the surface of an agar plate in a series of parallel lines. The loop is sterilized and then dragged across the surface of the agar in a perpendicular direction, which spreads the bacteria out in a thin layer. This process is repeated several times, creating a pattern of decreasing bacterial density on the plate. As the bacteria are spread out, they are forced to grow in isolated colonies, allowing for the identification and isolation of individual colonies of bacteria. The isolated colonies can then be used for further analysis.

The inoculated plates were kept for incubation for 24 hours at room temperature for ZMA and rest plates at 37℃. After this, colonies with characteristic colors, sizes, and forms were picked. They were transferred to newly prepared ZMA plates using the streak plate method, and purified by 3 rounds of repeated streaking. The pure bacterial isolates were then maintained on ZMA slants at 4^{o}C for further analysis.

3.6.2: Subculturing of plastisphere isolates

The main principle behind subculturing is to maintain the purity and viability of a microbial culture for further study and experimentation. It involves using aseptic techniques to prevent contamination and ensure that the subculture only contains the desired microorganisms. The process typically involves transferring a small amount of the original

culture (the inoculum) to a sterile medium in a new container, such as a petri dish or test tube, and incubating it under appropriate conditions to allow the microorganisms to grow.

3.7: Crystal Violet Assay to determine adherence of bacterial isolates to plastic.

Applying crystal violet dye, which binds to proteins and DNA, to adherent cells is one easy way to identify continued adherence of cells. The culture's level of crystal violet staining in a culture decreases when cell die because they become less adherent and disappear from the cell population.

To check the adherence property of plastisphere culture to plastic, sterile 24-well microtitre plates were used (Fig. 3.10). Microtitre plates are used in laboratories. They are mostly composed of plastic (POLYSTYRENE) and have many wells (cavities) that are spaced apart from one another. To carry out Crystal Violet Assay, 24 wells cell culture sterile microtitre plates were used.

The Crystal Violet Assay was done as per Bhangui et al. (2017). Two ml of Zobell Marine Broth (ZMB) was added to sterile wells of 3 sterile microtitre plates. A loopful of culture from the slants was inoculated into 3 sterile microtitre plates, and incubated at RT and 30^{o}C for marine bacteria and pathogens respectively. 500µl of ZMB broth was added to 3 sterile microtitre plates and to this 500µl of 24-h-old cultures were added. ZMB (1 ml) was kept as control in sterile microtitre plate and plates were incubated for 24 hours as detailed above. The culture broth microtitre plates were drained and unbound cells were removed by gently washing the micrititre plates with Phosphate Buffered Saline (PBS). Then gently washed twice with 1ml sterile distilled water, followed by drying of the microtitre plate by inverting on tissue paper for 15-30min. Then, 1ml of 0.1% of crystal violet solution was added to each well and kept for 45 mins at room temperature. Excess dye was washed off by

rinsing gently with 1ml sterile distilled water two to three times. The plates were dried and then 2ml of 30% acetic acid was added into each well to extract Cystal Violet. OD was taken at 600nm using UV-Vis spectrophotometer. 30% acetic acid was kept as a blank.

Figure 3.10: 24 well microtitre plates containing bacterial cultures.

CHAPTER 4: ANALYSIS AND CONCLUSIONS

4.1: Analysis

Table 4.1: Physicochemical parameters at the study areas.

Sr. No.	Study Site	Temperature	Salinity	pH
1.	Miramar	26	33	7
2.	Calangute	26	35	7

The physicochemical parameters found on both the study sites, Miramar and Calangute had no much difference. Temperature and pH values for both beaches are same. Salinity value is slightly differing for Miramar and Calangute beach (Table 4.1).

Table 4.2: The number of bacterial cultures obtained from the selected location sites.

Sr. No.	Location Site	No. of Bacterial Isolates
1	Miramar beach	16
2	Calangute beach	19

A total of 35 bacterial cultures were isolated from both the study areas. These includes: 16 cultures isolates from Miramar beach and 19 cultures isolates from Calangute beach. (Table 4.2; Fig. 4.1).

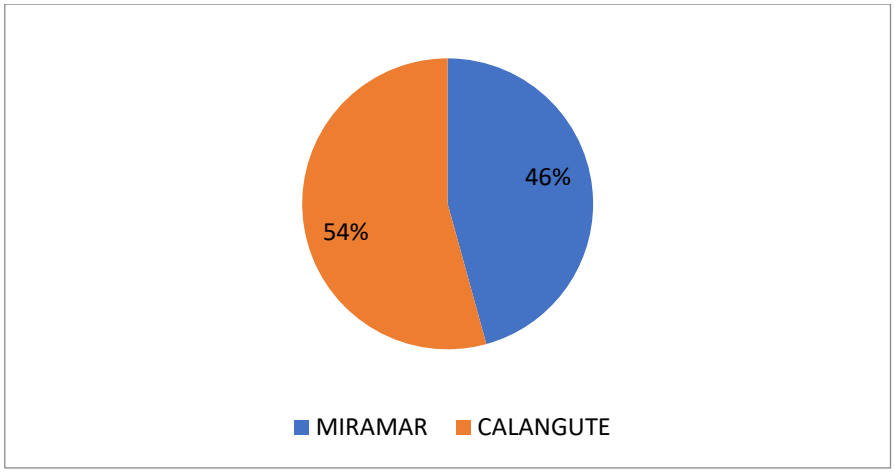

Fig 4.1: Proportion of Plastisphere Bacterial Isolates with respect to the different study sites.

Of all the bacterial cultures obtained from the Miramar beach plastic debris samples, 3 cultures were isolated on ZMA, whereas the rest (16) cultures were isolated on the rest of the media for pathogens (Table 4.3; Figs. 4.2-4.3). Of the bacterial cultures obtained from the Calangute beach plastic debris samples, 5 cultures were isolated on ZMA, whereas the rest (14) cultures were isolated on the rest of the media for pathogens (Table 4.4; Figs. 4.4-4.5).

Table 4.3: No. of bacterial isolates obtained from Miramar beach plastic debris samples.

Media	No. of bacterial isolates obtained
ZMA	3
MSA	2
EMB	4
XLD	4
TCBS	3

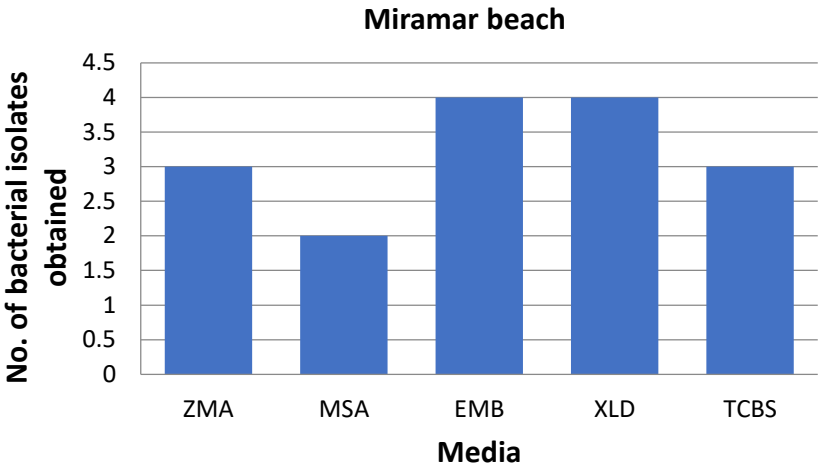

Figure 4.2: No. of bacterial isolates obtained on different media from the Miramar beach plastic debris samples.

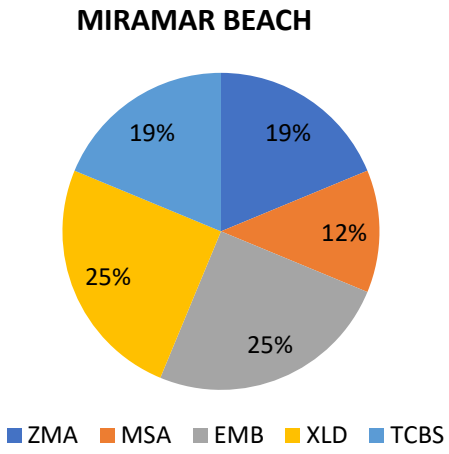

Figure 4.3: Proportion of bacterial isolates from Miramar beach plastic debris samples on different media.

Table 4.4: No. of bacterial isolates obtained from Calangute beach plastic debris samples.

Media	No. of bacterial isolates obtained
ZMA	5
MSA	7
EMB	4
XLD	2
TCBS	1

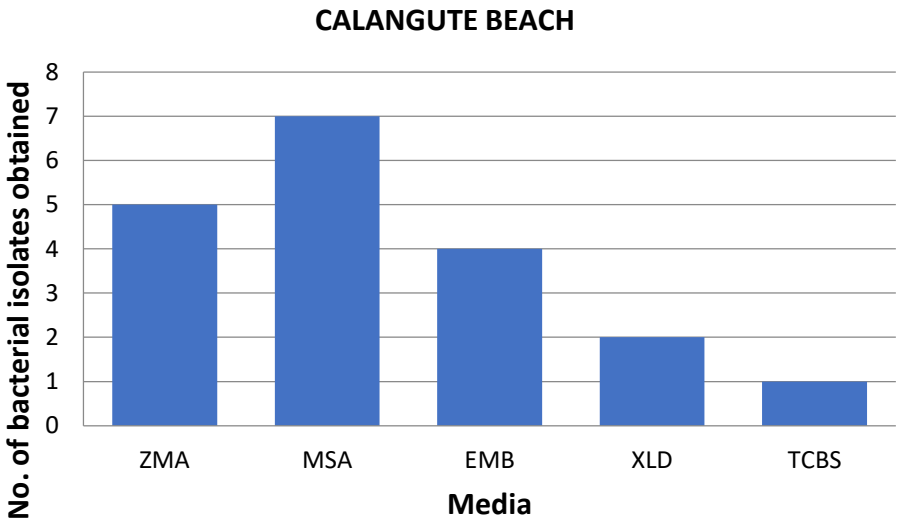

Figure 4.4: No. of bacterial isolates obtained on different media from the Calangute beach plastic debris samples.

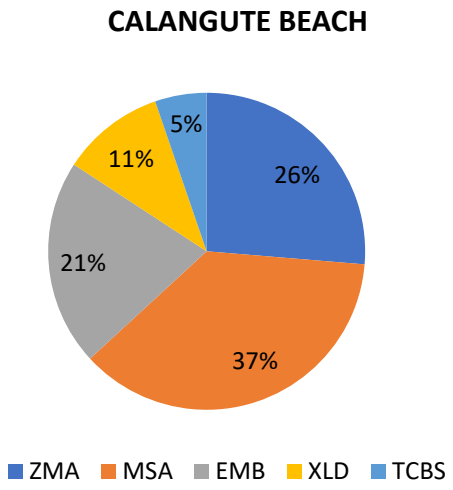

Figure 4.5: Proportion of bacterial isolates from Calangute beach plastic debris samples on different media.

Table 4.5: Plastisphere bacterial isolates obtained on both study sites

Microorganisms	Miramar beach	Calangute beach
S. aureus	–	–
E. coli	–	–
Salmonella	+	–
Shigella	–	–
Vibrio cholerae-like organisms (VCLO)	+	+
Vibrio parahaemolyticus-like organisms (VPLO)	+	+

Table 4.6: Sample-wise plastisphere bacterial isolates obtained from Miramar beach plastic debris samples.

Microorganisms	S1	S2	S3	S4	S5
S. aureus	–	–	–	–	–
E. coli	–	–	–	–	–
Salmonella	–	–	–	–	+
Shigella	–	–	–	–	–
Vibrio cholerae-like organisms (VCLO)	–	–	+	+	–
Vibrio parahaemolyticus-like organisms (VPLO)	+	–	–	–	+

Table 4.7: Sample-wise plastisphere bacterial isolates obtained from Calangute beach plastic debris samples.

Microorganisms	S1	S2	S3	S4	S5
S. aureus	–	–	–	–	–
E. coli	–	–	–	–	–
Salmonella	–	–	–	–	–
Shigella	–	–	–	–	–
Vibrio cholerae-like organisms (VCLO)	–	+	–	–	–
Vibrio parahaemolyticus-like organisms (VPLO)	–	–	–	+	–

Different plastisphere bacterial isolates were obtained on both study sites Miramar and Calangute beach (Table 4.5). The pathogens – VCLO and VPLO were noticed in both the study site plastic debris samples (Table 4.5). whereas *Salmonella* was obtained only in the Miramar beach plastic samples, specifically sample 5 (Table 4.6). *S. aureus, E. coli* and *Shigella* species were below detectable levels in all the samples from both the study areas (Tables 4.6-Table 4.7). VCLO and VPLO were recovered from 2 of the 5 plastic debris samples from Miramar (Table 4.6). Considering the Calangute plastic debris samples, VCLO and VPLO were recorded from only 1 of the 5 samples (Table 4.7)

Cystal Violet Assay

Table 4.8: Crystal violet assay for plastic debris associated bacterial isolates from Miramar beach.

Plastic samples	O.D. at 660nm
Control	0.0533
S2 ZMA	0.1213
S3 ZMA	0.0729
S4 ZMA	0.0653
S1 MSA	0.2369
S2 MSA	0.710
S4 EMB1	0.0653
S4 EMB2	0.1329
S5 EMB1	0.3340
S5 EMB2	0.1061
S4 XLD1	0.3112
S4 XLD2	0.0519
S4 XLD3	0.1373
S5 XLD	0.532
S1 TCBS	0.2250
S4 TCBS	0.1249
S5 TCBS	0.1123

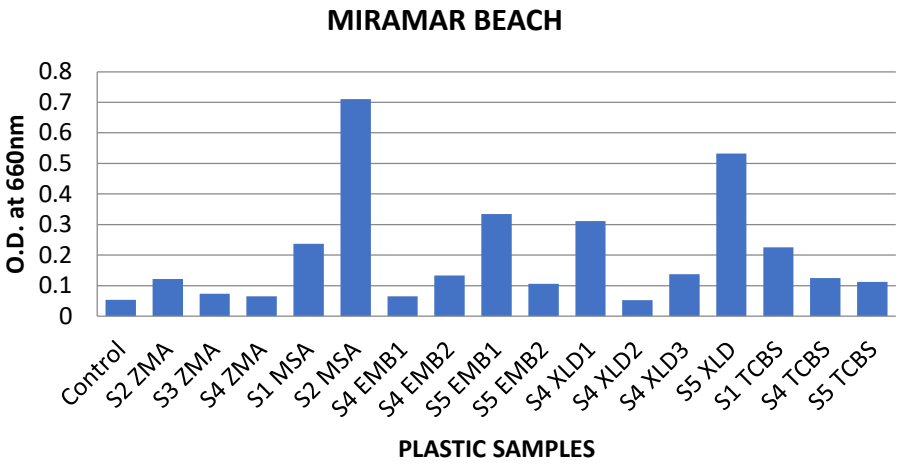

Figure 4.6: Crytal violet assay showing O.D. at 660nm for plastic samples (Miramar beach)

Table 4.9: Crystal violet assay for plastic debris associated bacterial isolates from Calangute beach

Plastic samples	O.D. at 660nm
Control	0.0346
S1 ZMA	0.1432
S2 ZMA	0.0875
S3 ZMA	0.1329
S4 ZMA	0.1365
S5 ZMA	0.0324
S1 MSA1	0.1361
S1 MSA2	0.0963
S2 MSA	0.1136
S3 MSA	0.0768
S4 MSA1	0.2218
S4 MSA2	0.1129
S5 MSA	0.0629
S2 EMB	0.1079
S4 EMB1	0.0595
S4 EMB2	0.2734
S4 EMB3	0.1763
S2 XLD	0.0853
S4 XLD	0.2102
S4 TCBS	0.1974

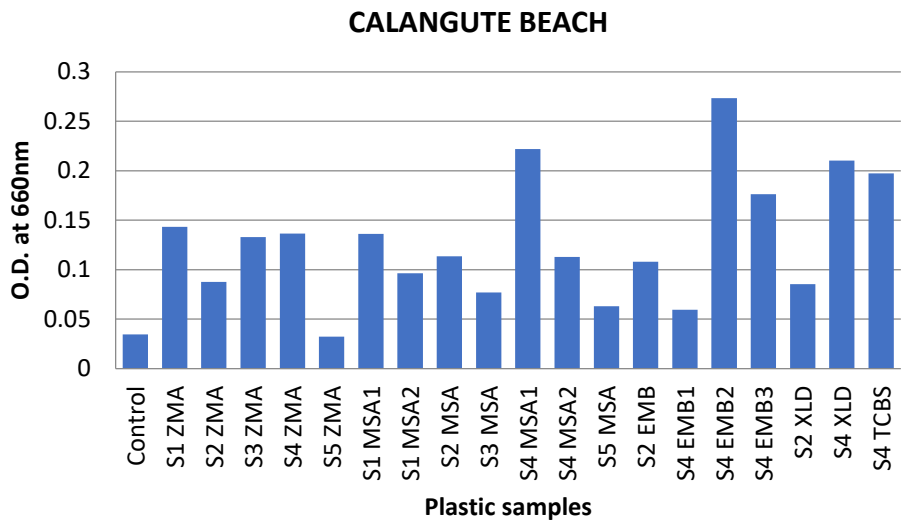

Figure 4.7: Crytal violet assay showing O.D. at 660nm for plastic samples (Calangute beach)

Crystal violet assay for plastic debris associated bacterial isolates to check adherence of plastisphere bacteria on plastic. Plastisphere bacteria from S1 MSA, S2 MSA, S4 XLD, S4 EMB shows strong adherence to the plastic from Miramar beach and plastisphere bacteria from S4 MSA, S4 EMB, S4 XLD shows adherence to the plastic from Calangute beach.

4.2: Discussion

My present study was conducted on microorganisms associated with plastic marine debris, especially the attachment of the human pathogens in plastisphere. The study areas selected were 2 popular beaches of Goa, Miramar and Calangute beach. Different plastic samples like milk packets, plastic bags were collected from both beaches which were found in moist soil. The physicochemical parameters were found to be almost same for both beaches. A total of 35 plastisphere bacterial isolates were isolated from both beaches. Among which, more number of plastisphere bacterial isolates were found from Calangute beach as

compared to Miramar beach. On EMB and XLD, more number of bacterial isolates were found from Miramar beach and on MSA media, more number of bacterial isolates were found from Calangute beach as compared to the other media.

From both beaches, human pathogens associated to plastics were found. *Vibrio cholerae* like organisms (VCLO) and *Vibrio parahaemolyticus* like organisms (VPLO) isolated on TCBS media were found from both beaches. *Salmonella* isolated on XLD media were found from Miramar beach. *Vibrio cholerae* like organisms (VCLO) were found on 2 plastic samples out of 5 plastic samples and *Vibrio parahaemolyticus* like organisms (VPLO) were found on 2 plastic samples out of 5 plastic samples collected from Miramar beach. *Vibrio cholerae* like organisms (VCLO) and *Vibrio parahaemolyticus* like organisms (VPLO) was found on 1 plastic sample out of 5 plastic sample collected from Calangute beach. Crystal violet assay for plastic debris associated bacterial isolates was done to check the adherence of plastisphere bacteria on plastic using sterile microtitre plates. *S. aureus* from MSA, *E. coli* from EMB and *Salmonella* from XLD showed strong adherence to the polystyrene microtitre plates.

The most common pathogenic species of *Vibrio* bacteria that cause diseases in humans include:

Vibrio cholerae: Responsible for causing cholera, a severe diarrheal illness transmitted through contaminated food or water. Cholera can lead to dehydration and, if left untreated, can be fatal. *Vibrio parahaemolyticus*: Causes vibriosis, which can result from consuming raw or undercooked contaminated seafood. It can lead to gastroenteritis or primary septicemia.

Vibrio vulnificus: Another pathogenic species causing vibriosis, often acquired through exposure to seawater or consumption of contaminated seafood. *V. vulnificus* infections can

lead to wound infections that progress rapidly to septicemia, especially in individuals with underlying health conditions. These species of *Vibrio* bacteria are known for their pathogenicity and ability to cause significant health issues in humans. Understanding the characteristics and clinical implications of these common pathogenic *Vibrio* species is essential for effective diagnosis, treatment, and prevention of *Vibrio*-related diseases (Austin et al., 2018)

Recent sequencing results suggest that microplastics can act as vectors for the dispersal of potentially pathogenic *Vibrio* spp. in marine environments (Kirstein et al., 2016). Microplastics can be transported over long distances, allowing for the spread of harmful or even human pathogenic species. A study analyzed plastics and water samples from the North and Baltic Sea for potentially human pathogenic *Vibrio* spp. *Vibrio parahaemolyticus* was found on microplastic particles like polyethylene, polypropylene, and polystyrene. This highlights the need for detailed biogeographical analyses of marine microplastics (Kirstein et al., 2016).

The study of microbial communities on plastic marine debris has identified *Vibrio* bacteria, such as *V. harveyi*, *V. natriegens*, and *V. alginolyticus*, on plastic samples like polypropylene (Zettler et al., 2013). These bacteria are known for their fast growth rates and diverse metabolic capabilities, including nitrogen fixation. The presence of these bacteria on plastic samples, especially in higher concentrations than in natural environments, suggests a unique microbial community structure in the Plastisphere. This finding underscores the ecological implications of plastic pollution on marine ecosystems, including the potential for plastic to serve as a vector for infectious diseases. Given that fishes and birds both consume PMD and that human pathogenic *Vibrio* strains have been detected in fish, plastic may act as a vector for infectious diseases. PMD may travel great distances and has been demonstrated to carry invasive species because it remains longer than natural substrates like feathers, wood,

and macroalgae. The presence of *Vibrio* bacteria in the Plastisphere highlights the complex interactions between microbial communities and plastic marine debris, emphasizing the need for further research to understand the ecological consequences of plastic pollution in our oceans (Zettler et al., 2013).

Vibrio cholerae is a bacterium responsible for causing cholera, an acute diarrheal infection transmitted through contaminated food or water (woodford et al., 2024). There are two main serogroups of *V. cholerae* associated with cholera outbreaks: O1 and O139. The O1 serogroup is further categorized into two biotypes: classical and El Tor. The El Tor biotype is known for displacing the classical biotype as the predominant epidemic strain and is responsible for the ongoing seventh pandemic of cholera. *V. cholerae* has the ability to form biofilms in various environments, allowing it to survive outside the host and persist in aquatic environments, potentially leading to the spread of cholera. The bacterium can associate with a variety of surfaces, including plastic waste, and has been shown to resuscitate from a viable but nonculturable state under certain conditions, contributing to the seasonal nature of cholera epidemics. Understanding the environmental persistence and transmission dynamics of *V. cholerae* is crucial for effective public health management strategies to prevent and control cholera outbreaks (Woodford et al., 2024).

Vibrio bacteria are known to cause diseases in humans, with *Vibrio cholerae* being the most well-known species responsible for cholera (Austin et al., 2018). Cholera is a severe diarrheal illness that can be fatal if not promptly treated with rehydration therapy, antibiotics, and nutritional supplements. Other pathogenic *Vibrio* species include *Vibrio parahaemolyticus, Vibrio vulnificus,* and Vibrio alginolyticus, which can cause vibriosis through contaminated seafood consumption or direct exposure to water. *Vibrio vulnificus,* in particular, is an opportunistic pathogen that can lead to severe wound infections and

septicemia, especially in individuals with underlying diseases such as liver conditions, diabetes, or malignancies.

The incidence of *Vibrio spp*. infections, including cholera and vibriosis, is on the rise, possibly due to factors like climate change and increasing sea water temperatures. These infections can present with various clinical manifestations, ranging from mild gastroenteritis to severe systemic infections, depending on the specific *Vibrio* species, route of infection, and host susceptibility. Proper management of *Vibrio* infections involves supportive care, rehydration therapy, antibiotics, and in some cases, surgical interventions like debridement of infected tissues (Austin et al., 2018).

Overall, *Vibrio* bacteria pose a significant public health concern, and understanding their pathogenicity, transmission routes, and clinical implications is crucial for effective prevention and management of *Vibrio*-related diseases (Austin et al., 2018). The seasonal distribution of Vibrio spp. infections is mostly determined by environmental factors, including temperature rise, precipitation patterns, and the introduction of *Vibrio* pathogens into aquatic reservoirs. It is believed that part of the seasonal pattern of illnesses is caused by the brief bloom of *Vibrio* spp. in aquatic habitats during warm months. Estuaries and coastal waters are among the aquatic settings that are conducive to the growth and multiplication of *Vibrio* bacteria due to elevated temperatures and increased rainfall. Because of this, there's a higher chance of coming into contact with *Vibrio* spp. during these times, which increases the likelihood of infections in vulnerable groups. Comprehending the environmental conditions that impact the occurrence and transmission of *Vibrio* spp. infections is crucial, as seen by the seasonal distribution of these diseases (Austin et al., 2018).

Non-typhoidal salmonella infections can cause various diseases, including typhoid and paratyphoid fever, salmonella enterocolitis, and invasive non-typhoidal salmonella disease (Sarkar et al., 2019). Typhoid and paratyphoid fever caused 14.3 million cases and

136,000 deaths in 2017, with a higher case fatality rate compared to non-typhoidal salmonella invasive disease. Salmonella enterocolitis, a type of non-invasive salmonella infection, caused 95.1 million cases and 50,800 deaths in 2017, with similar numbers of deaths and disability-adjusted life years. Invasive non-typhoidal salmonella disease is most common in sub-Saharan Africa, where specific serovars and sequence types of salmonella are endemic. The study emphasizes the importance of investigating sources and transmission pathways of non-typhoidal salmonella invasive disease to implement effective preventive and control measures. The burden of non-typhoidal salmonella invasive disease is significant, especially in children, the elderly, individuals with HIV infection, and areas with low socioeconomic development. Additional studies are needed to determine the contribution of risk factors such as malaria, acute malnutrition, and sickle cell disease to the burden of non-typhoidal salmonella invasive disease (Sarkar et al., 2019).

4.3 Conclusions

The findings of this preliminary study raise concerns regarding the potential transmission of harmful diseases via plastic debris, which can serve as a vector for pathogen dispersal over long distances. The implications of these findings extend beyond environmental conservation to include public health considerations, highlighting the urgent need for mitigation strategies to address plastic pollution and its associated risks to both marine ecosystems and human populations.

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APPENDICES

Media composition

Zobell Marine Agar (ZMA)

Ingredients	gm/L
Peptone	5
Yeast extract	1
Ferric citrate	0.100
Sodium chloride	19.45
Magnesium chloride	8.80
Sodium sulphate	3.240
Calcium chloride	1.800
Potassium chloride	0.550
Sodium bicarbonate	0.160
Potassium bromide	0.080
Strontium chloride	0.034
Boric acid	0.022
Sodium silicate	0.004
Sodium fluorate	0.0024
Ammonium nitrate	0.0016
Disodium phosphate	0.008
Agar	15
Distilled water	1000Ml
pH	7.5 -7.77

Mannitol Salt Agar (MSA)

Ingredients	Grams /Litre
Protease peptone	10.000
HM peptone	1.000
Sodium chloride	75.000
D-Mannitol	10.000
Phenol red	0.025
Agar	15.000
pH	7.4±0.2

Eosin Methylene Blue (EMB) Agar

Ingredients	Grams/litre
Distilled water	1.000
Peptone	3.000
Lactose	5.000
Saccharose (Sucrose)	5.000
Dipotassium hydrogen phosphate	2
Eosin- Y	0.40
Methylene blue	0.065
Agar	15

Xylose Lysine Deoxycholate (XLD) Agar

Ingredients	Grams/litre
Xylose	3.500
L-Lysine	5.000
Lactose monohydrare	7.500
Sucrose	7.500
Sodium chloride	5.000
Yeast extract	3.000
Phenol red	0.080
Sodium deoxycholate	2.500
Sodium thiosulphate	6.800
Ferric ammonium citrate	0.800
Agar	13.500
Ph	7.4 ±0.2

Thiosulfate- Citrate- Bile Salts Sucrose (TCBS) Agar

Ingredients	Grams/litre
Distilled water	1.000
Yeast extract	5.000
Peptic digest of animal tissue	10.000
Sodium citrate	10.000
Sodium thiosulphate	10.000
Sodium cholate	3.000
Oxgall	5.000
Sucrose	20.000
Sodium chloride	10.000
Ferric citrate	1.000
Bromothymol blue	0.40
Thymol blue	0.40
Agar	15.000

Zobel Marine Broth (ZMA)

Ingredients	gm/L
Peptone	5
Yeast extract	1
Ferric citrate	0.100
Sodium chloride	19.45
Magnesium chloride	8.80
Sodium sulphate	3.240
Calcium chloride	1.800
Potassium chloride	0.550
Sodium bicarbonate	0.160
Potassium bromide	0.080
Strontium chloride	0.034
Boric acid	0.022
Sodium silicate	0.004
Sodium fluorate	0.0024
Ammonium nitrate	0.0016
Disodium phosphate	0.008
Distilled water	1000 mL
pH	7.5-7.7

Preparation of 1% Crystal Violet

Ingredient	Grams/Litre
Crystal Violet	0.1
Distilled Water	100

Prepration of 30% Acetic acid

Add 30ml acetic acid in 70ml distilled water.