Studies on plastisphere bacteria associated with plastic marine debris

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By

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GOA UNIVERSITY APRIL 2023

Examined by: **Seal of the School**

DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, "**Studies on plastisphere bacteria associated with plastic marine debris"** is based on the results of investigations carried out by me in the M.sc Marine Microbiology at the School Of Earth, Ocean and Atmospheric Sciences, Goa University under the Supervision/Mentorship of Dr. Priya M. 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 be 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 plastisphere bacteria associated with plastic marine debris**" is a bonafide work carried out by Mr. ANIKET SINGH under my supervision/mentorship in partial fulfilment of the requirements for the award of Master Degree in the Discipline of M.sc Marine Microbiology at the School of Earth, Ocean and Atmospheric Sciences, Goa University.

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ANIKET SINGH

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INTRODUCTION

1. INTRODUCTION

Plastic pollution is a major global problem, with a staggering 370 million tonnes of plastic produced worldwide in 2019 alone (Plastics Europe, 2020). Despite efforts to recycle or incinerate 21% of the plastic waste generated, the majority of plastic waste is still released into the environment in some form or another (Yuan et al., 2020). The top five plastic polymers, including polyethylene, polypropylene, polyvinyl chloride, polystyrene, and polyethylene terephthalate, account for around 80% of the total plastic production (Saini et al.,2022).

1.1 WHAT ARE MICROPLASTICS?

The production of plastic objects encompasses a wide range of shapes and dimensions, including very tiny ones that measure less than 5 millimeters. These extremely small plastic pieces are commonly referred to as "microplastics"(Hortan et al.,2017). They can be either intentionally manufactured or can result from the breakdown of larger plastic products, such as bags, bottles, or packaging materials, etc. Microplastics are a growing environmental concern as they can accumulate in waterways, oceans, and soil, posing a threat to aquatic and terrestrial organisms and ecosystems. Plastic waste introduced into the environment can experience various forms of deterioration, including physical, chemical, and biological processes. These processes can result in the fragmentation of larger plastic pieces into smaller particles, such as microplastics (MPs) and even nano plastics (NPs). (Ahmed et al., 2021;
Chen et al., 2019).

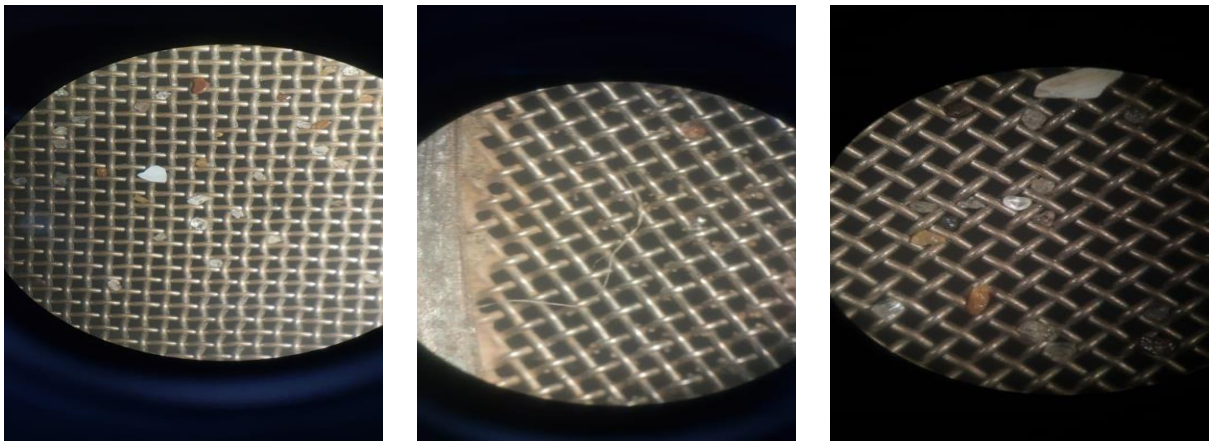

Fig 1: Microplastic fibres under the stereo microscope

1.2 HOW ARE MICROPLASTIC CLASSIFIED?

Microplastics can be classified based on their origin and size. Here are the most common classifications:

- Primary microplastics: These are intentionally produced microplastics, such as microbeads used in cosmetics, industrial abrasives, and pellets used in plastic manufacturing. (Hortan et al.,2017)

- Secondary microplastics: These are created when larger plastic debris is degraded into smaller pieces by physical, chemical, or biological processes. For example, plastic bags, bottles, and other plastic waste that enter the ocean can break down into smaller fragments. (Hortan et al.,2017)

1.3 SOURCES OF MICROPLASTIC WITHIN THE ENVIRONMENT

Plastic is introduced to the terrestrial environment through various sources, including vehicle tire abrasion and traffic (Kole et al. 2017; Evangeliou et al. 2020), household activities like the use of cleaning agents and cosmetics (Murphy et al. 2016), synthetic fibers from clothing and textile washing (Habib et al. 1998; Browne et al. 2011; Napper and Thompson 2016; Boucher and Friot 2017), coatings, paint, and preparatory painting activities like abrasive blasting (Takahashi et al. 2012; Song et al. 2015; Chae et al. 2015). Improperly managed waste, industrial spillages, and littering are also significant contributors of plastic to the terrestrial environment (Sadri and Thompson 2014; Lechner et al. 2014; Mason et al. 2016; Murphy et al. 2016; Kay et al. 2018; Hale et al. 2020 and references therein). Additionally, burning plastics through uncontrolled disposal methods or natural wildfires can release plastic particles into the atmosphere and surrounding environment, eventually making their way into waterways (Gullett et al. 2007; Asante et al. 2016; Ni et al. 2016; Hale et al. 2020). Plastic products: Microplastics can be generated from the degradation of larger plastic products such as water bottles, plastic bags, and fishing gear. Synthetic textiles like polyester, nylon, and acrylic release microfibers when washed. These fibers can end up in waterways and oceans. Microbeads, which are tiny plastic beads used in personal care products like toothpaste, exfoliants, and scrubs, can also end up in waterways and oceans. (Hortan et al.,2017)

1.4 THE MICROPLASTIC MENACE: A GROWING GLOBAL CONCERN

The presence of microplastics is a growing global concern as they are found in both aquatic and terrestrial food webs, including commercially important species consumed by humans, such as zooplankton, bivalves, crustaceans, fish, and other marine vertebrates. Plastic waste in the marine environment undergoes degradation and fragmentation into microplastics due to physical and chemical factors like ultraviolet radiation, physical abrasion, and chemical oxidation. Plastic production globally in 2019 amounted to 368 million tons (Agostini et al.,2021). The adhesion of harmful microorganisms to plastic waste was initially noticed by (Maso et al.,2014).

A growing amount of plastic is entering the aquatic environment due to the high manufacturing rates, low recycling rates, and poor waste management practices for plastics. It is projected that the world will produce 1.1 billion tonnes of primary plastic by 2050 if nothing is done to slow the upward trend in the manufacture and consumption of plastic goods (Geyer, 2020). Due to COVID-19's effects on the sector, plastic production in 2020 fell by about 0.3% compared to 2019. (Tiseo, 2021). Although, governments have made ambitious commitments, it is projected that annual emissions could reach 53 million metric tons by the year 2030. (Borrelle et al., 2020).

Plastic waste introduced into the environment can experience various forms of deterioration, including physical, chemical, and biological processes. These processes can result in the fragmentation of larger plastic pieces into smaller particles, such as microplastics (MPs) and even nano plastics (NPs). (Ahmed et al., 2021; Chen et al., 2019). Mechanical abrasion of plastics, known as fragmentation, occurs when plastics collide with hard rocks in sediments due to the impact of tides and wind waves, causing them to experience mechanical weathering, making them more susceptible to photodegradation and biodegradation. On the other hand, the degradation of plastics involves photo-oxidation, thermal-oxidation, hydrolysis, and

biodegradation processes. These mechanisms are interconnected and can impact each other. When plastics are introduced into aquatic environments, microorganisms quickly adhere to the plastic surface and form a stable biofilm within 7 days (Zettler et al., 2013). This plastic debris can also absorb various chemical substances present in the surrounding water (Ogata et al., 2009; Rochman et al., 2014). The absorption process is affected by plastic properties such as type, size, and surface characteristics (Koelmans et al., 2016). The presence of plastic additives and organic pollutants, such as phthalates, bisphenols, and terephthalic acid, has been detected in plastics (Kumar et al., 2022). Several studies have investigated the accumulation mechanism of persistent organic pollutants (POPs) on plastic debris in aquatic environments. These studies have looked at various pollutants, including per fluorooctanesulfonate (PFOS) (Wang et al.,2015), per fluorooctanesulfonamide (PFOSA) (Wang et al.,2015), polycyclicaromatic hydrocarbons (PAHs) (Chen et al., 2020; Pittura et al.,2018; Sørensen et al.,2020; Tang et al.,2018; Wang et al.,2018), per and polyfluoroalkyl substances (PFAS) (Ateia et al.,2020; Llorca et al.,2018), tetracycline, and dichlorodiphenyltrichloroethane (DDT) (Wang et al.,2018). The accumulation of organic substances on virgin plastics occurs through two mechanisms, namely van der Waals force and self-accumulation of organic pollutants. However, in the case of biofilm-covered plastics, the primary mechanism is hydrophobic interactions, which provide a more potent force for contaminant adsorption than cationic exchange. This has been observed in studies on the accumulation of contaminants such as PFAS, tetracycline, and

DDT (Ateia et al., 2020; Bhagwat et al., 2021; Llorca et al., 2018; Wang et al., 2015). Additionally, the hydrophobicity and large specific surface area of plastics enable them to carry heavy metals (as noted by Liu et al., 2021; Rochman et al., 2014; Zhang et al., 2021), organic pollutants (as mentioned by Bhagwat et al., 2021; He et al., 2022), and antibiotics (as reported

by Dussud et al., 2018b; Eckert et al., 2018), which can affect the occurrence, migration, and transportation of water pollutants.

The formation and colonization of biofilms on plastic surfaces can also impact the accumulation of contaminants in aquatic ecosystems. Biofilms are communities of microorganisms that form complex structures and attach to solid surfaces while being enclosed in an extracellular polysaccharide matrix. A significant amount of microplastic particles in rivers come from sewage effluent. Research suggests that the plastisphere communities attached to these particles differ from the surrounding environment downstream of the effluent. As a result, plastic properties can be altered due to the attachment of biofilm communities, which further affects the accumulation mechanism of pollutants in aquatic environments (Rummel et al.,2017).

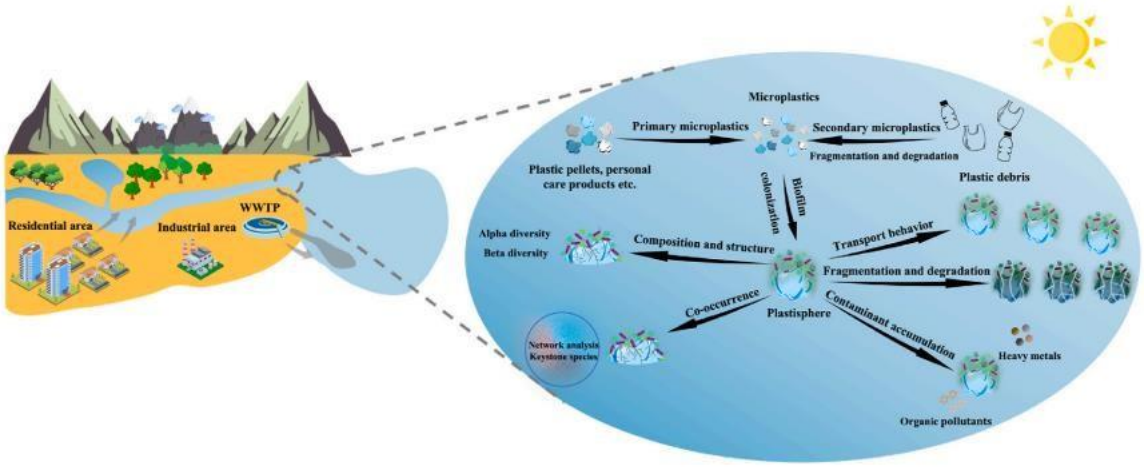

Fig 2: The source and fate of aquatic plastic waste, and the formation of the aquatic plastisphere. Source: (Yue et al., 2022)

Additionally, plastics have been found to serve as a habitat for microbial colonization and biofilm formation. Mincer et al. (2016) estimated that the plastisphere biofilms around the world contain approximately 1,000 to 15,000 tons of microorganisms.

1.5 IMPACT OF MICROPLASTIC ON HUMAN HEALTH

Microplastics have been found to contaminate salt (Seth and Shriwastav, 2018), drinking water (Pivokonsky et al., 2018), bottled water, beer (Wiesheu et al., 2016), mussels (Berglund et al., 2019) and fish (Neves et al., 2015). Once microplastics are ingested, they pass through the gastrointestinal tract and reach the intestine. Only microplastics that penetrate the intestinal mucus or have a biocompatible coating are able to reach intestinal cells. These microplastics can be taken up by specialized M-cells (Ensign et al.,2012) or through paracellular transference (Volkheimer,1977), which may lead to the systemic circulation (Eyles et al.,1995). In vitro studies suggest that microplastics can affect gene expression, cell viability, and pro-inflammatory responses in cells (Forte et al.,2016). In vivo studies on mice showed that even low concentrations of microplastics can cause a decrease in spermatogenic cells and sperm count after 42 days of exposure (Xie et al.,2020). Interestingly, the amount of microplastics ingested by humans per week is estimated to be much higher than the amount that causes adverse effects in mice (Senathirajah and Palanisami,2019).

Dangerous microorganisms can be present on the surface of microplastics and take benefit of erosion, ulcer and fissures resulting from these particles to cause infections. For instance, Vibrio spp. bacteria mainly part of foodborne diseases was present on microplastics in the marine environment (Kirstein et al., 2016) (Letchumanan et al., 2019). These are in addition to the pollutants adsorbed on the plastic surface, which can also affect human health.

1.6 WHAT IS PLASTISPHERE?

The Plastisphere is a diverse community of microorganisms that includes heterotrophs, autotrophs, predators, and symbionts. This community is unique and distinct from the surrounding surface water, suggesting that plastic debris provides a new ecological niche in the

open ocean. The term "Plastisphere" was first introduced by (Zettler et al.,2013), who conducted a thorough analysis of microbial communities on plastic debris. They used nextgeneration sequencing and scanning electron microscopy (SEM) to examine three polyethylene and three polypropylene plastic pieces, which were approximately 2-20 mm long and collected from offshore waters of the North Atlantic (Ressier et al.,2014). Due to its longer half-life compared to natural marine substrates and hydrophobic surface that facilitate microbial colonization and biofilm formation, plastic debris is different from other substrates found in the ocean's upper layers (Zettler et al.,2013).

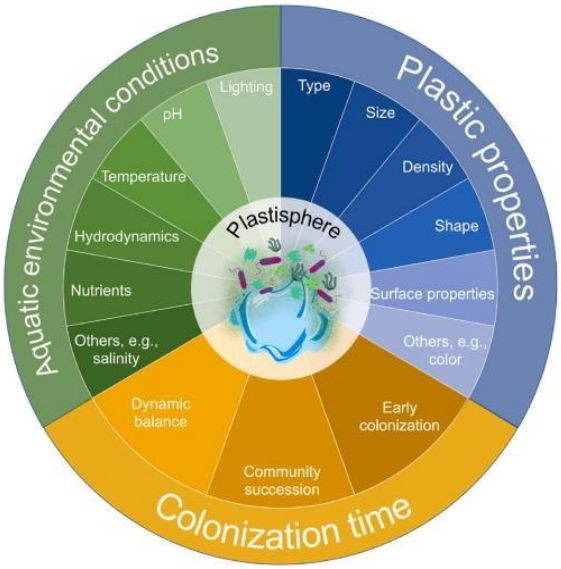

Fig 3: The Impact factors on the development of aquatic plastisphere (Source: Yue et al., 2022).

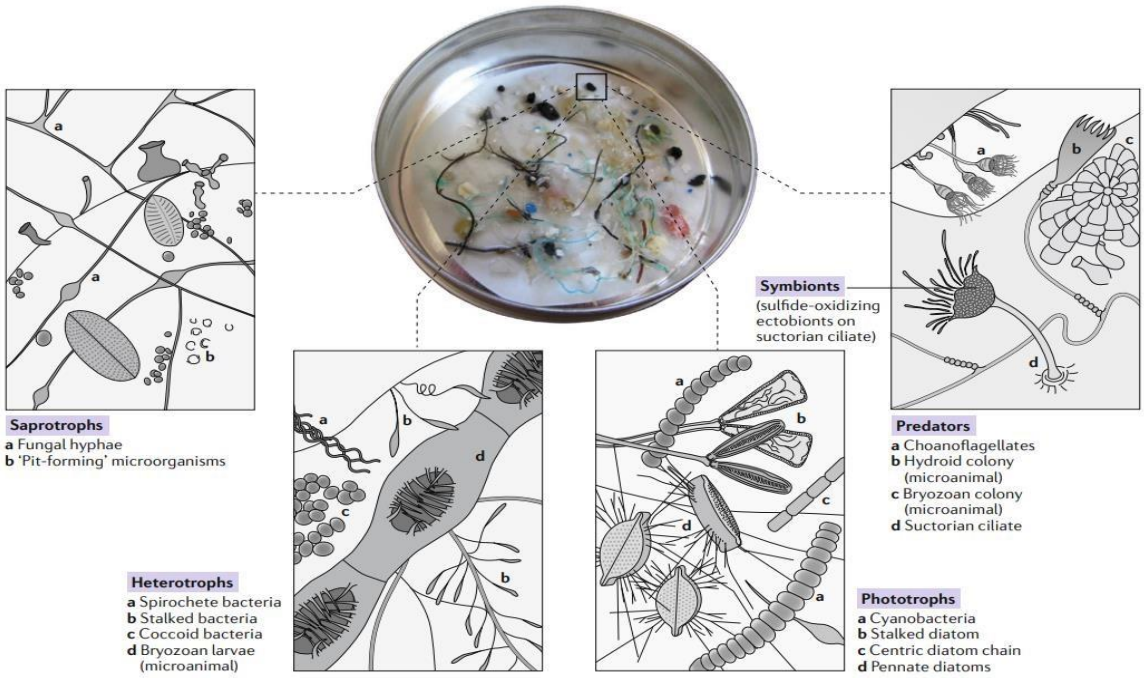

Fig 4: The Plastisphere communities. It shows a microbial ecosystem of bacteria, Protista and animals on plastic substrate in an oligotrophic open ocean. Cyanobacteria, diatoms, Predatory ciliates and hydroids, grazers including ciliates and bryozoans, symbiotic relationships and heterotrophs are the members. Source: (Zettler et al., 2020)

According to studies by Amaral-Zettler et al. (2020), De Tender et al. (2015), Kirstein et al. (2019), and Zettler et al. (2013), diatoms such as pennate diatoms, stalked diatoms, and centric diatom chains, as well as bacteria like Cyanobacteria and Alphaproteobacteria including Erythrobacter and Roseobacter genera, are considered early colonizers in the plastisphere. The history of synthetic polymers dates back to the 1850s and 60s when Parkesine, also known as celluloid, was created. Bakelite, the first completely synthetic polymer, was then invented in 1907. Polyvinyl chloride (PVC), the first modern plastic, was developed in the 1920s and made more functional by incorporating additional substances to enhance its malleability (Geyer et al.,2020). Additionally, the plastic's properties, including type, size, and surface characteristics, can influence the selective formation of initial microbial communities, as suggested by studies by (Min et al.,2020) and (Pinto et al.,2019). Moreover, when plastics are transported in aquatic ecosystems, they can come into contact with pollutants and nutrients and serve as carriers,

potentially affecting the biogeochemical cycle, as observed in studies by Miao et al. (2021); Rogers et al. (2020) and Rummel et al. (2017). The movement of plastics in aquatic settings, including settling and rising, is affected by their degradation and fragmentation processes. These processes involve the breaking down of plastics due to both abiotic and biotic factors, which cause them to lose their structural integrity (Moore, 2008).

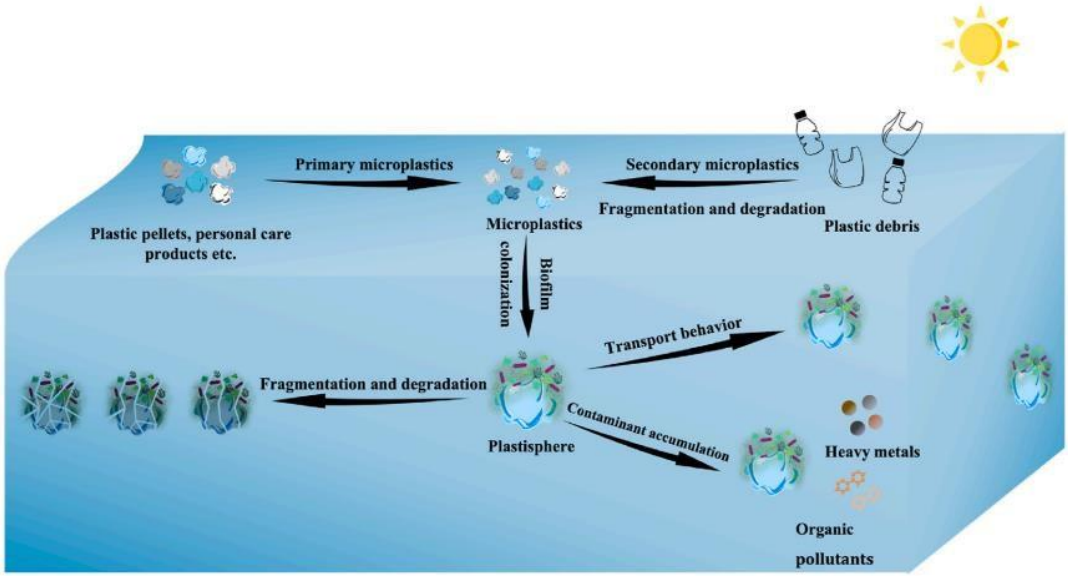

Fig 5: The cross section represents the water ecosystem where the environment behaviours of the plastisphere occur. Three environmental behaviours: Transport, Fragmentation and Contaminant accumulation are shown. In terms of contaminant accumulation, heavy metals, and organic pollutants are included. Source: (Yue et al., 2022)

1.7 ROLE OF PLASTISPHERE IN SPREAD OF ANTIBIOTIC RESISTANT BACTERIA AND ANTIBIOTIC RESISTANCE GENES

The Plastisphere has also been found to contain pathogenic species, raising concerns about the role of rivers in transporting these microbes. Around 5 to 13 million tonnes of plastic were estimated to have entered the ocean in 2010 (Jambeck, J. R. et al. 2015), leading to 15 to 51 trillion plastic particles floating in the marine environment (Van Sebille, E. et al 2015). If the current release rate persists, this amount of pollution could potentially double by 2030, according to the World Economic Forum, Ellen MacArthur Foundation, and McKinsey &

Company (2016). Microorganisms can colonize and thrive on plastic debris, creating a new ecosystem referred to as the plastisphere. This ecosystem is characterized by microbial biofilms, including potential pathogens and harmful algal bloom species, that grow on plastic debris and can be transported long distances. The term "plastisphere" originally referred to the life found on microplastics collected from the North Atlantic Subtropical Gyre, also known as the "garbage patch," but has since been used to describe the microbial communities associated with plastic debris in various aquatic environments. The microorganisms can biotransform plastic debris into chemicals that may pose a threat to human health and food security, according to (McCormick et al.,2011; Linda et al.,2020). According to research, more than 10,000 tons of plastic can be found floating in the open ocean (Cozar et al.,2014).

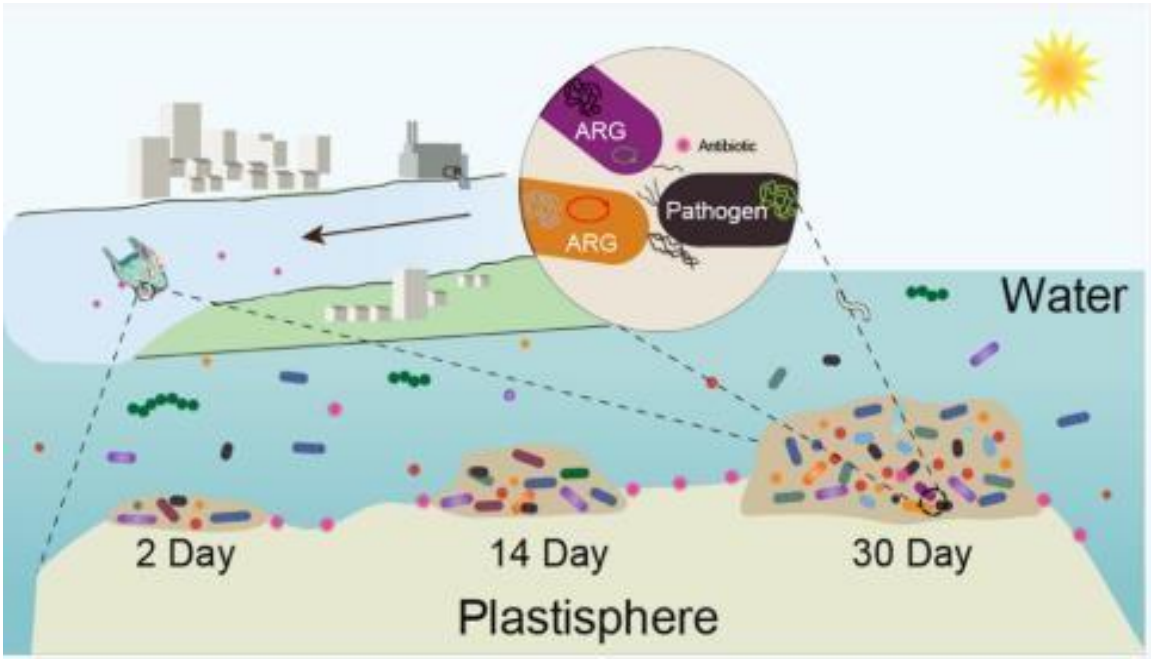

Fig 6: Plastic in marine environment harbouring antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARG) Source: (Yang et al., 2020)

Recent investigations have discovered that plastic in the marine environment can harbor antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARG) (Yang 2019 and

Moore, R. E.; 2020). Studies have also found that aquaculture systems that use plastic components can contain various ARBs and ARGs (Zhang, 2020). However, little is known about the occurrence and time-related changes of the antibiotic resistome in the development of microbial colonization and biofilm formation on plastic surfaces, such as the initial, intermediate, and persistent stages of antibiotic resistance in the plastisphere. The potential risks of plastisphere can be assessed from four perspectives: presence of pathogens, potential transfer of antibiotic resistance genes (ARGs) through Horizontal Gene Transfer (HGT), increased resistance to antibiotics, and transfer of these elements through the food web. Their research identified a total of 63 bacterial pathogens associated with human diseases in the plastisphere, and the proportion of pathogens in the plastisphere was significantly higher than that in the surrounding water, indicating a potentially greater health risk from the plastisphere than from water alone. Recent studies have shown that ARGs can also be transferred through the food chain, potentially contaminating aquatic ecosystems and threatening to human health (Zhu, 2019). The plastisphere is a major contributor to the acquisition and spread of antibiotic resistance and disease, as it facilitates the persistence of Mobile Genetic Elements (MGEs) and the enrichment of pathogenic bacteria. The Plastisphere's growth mode provides a protective environment for bacteria, allowing them to avoid antibiotic invasion and prolonging the existence of antibiotic resistance. This creates an opportunity for adaptive genetic changes through Horizontal Gene Transfer (HGT) or mutation, which could lead to long-term environmental risks for both the ecosystem and human health (Yang et al.,2020).

Chapter 2
AIMS AND OBJECTIVES

2. AIMS & OBJECTIVES

Microplastics are tiny plastic particles, less than 5mm in size, found in the environment. They can enter oceans through waterways, soil, and air, which can be ingested by wildlife and humans. Microplastics can have harmful effects on ecosystems and human health, thus, studying them is essential to understand and mitigate these impacts. Microplastics have been found in many food and water sources, including seafood, drinking water, and even bottled water. They can potentially accumulate in human organs and tissues, and their long-term health effects are not yet fully understood. Studying microplastics can help us understand the extent of the human health risks associated with exposure to these particles. It helps us understand how microplastics and their associated microbial communities can potentially impact human health. The plastisphere refers to the diverse community of microorganisms that colonize plastic debris in the environment, including in oceans, rivers, and soils. Some studies have suggested that the microbial communities on plastic debris can potentially promote the growth of harmful bacteria, including pathogens that can cause human diseases. Additionally, microplastics have been shown to accumulate toxins from the environment, which can potentially be transferred to humans through the food chain. Studying the plastisphere can provide insights into the types of microorganisms and toxins associated with microplastics, and how they may affect human health. For example, research has suggested that certain bacteria colonizing microplastics can produce antibiotic-resistant genes, which could be transferred to human pathogens and lead to increased antibiotic resistance in humans (Wang et al., 2021). Some pollutants associated with microplastics, such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs), have been linked to potential health effects in humans, such as cancer and reproductive problems (Pittura et al., 2018). Studying the sensitivity of plastisphere communities to these pollutants can help us better understand the potential risks to human health associated with exposure to microplastics and their associated

pollutants. Understanding the sensitivity of these communities to different pollutants can help us identify which pollutants may inhibit or promote plastic biodegradation, and inform strategies to enhance the biodegradation of plastics in the environment.

Thus, the aims and objectives of the present study were as follows:

Aim: To study the sensitivity of Plastisphere bacterial isolates to different classes of pollutants.

Objectives:

1. To isolate Plastisphere associated bacteria from plastic marine debris.
2. To investigate the sensitivity of Plastisphere bacterial isolates to different classes of pollutants.

Chapter 3

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 STUDY AREA:

The 1st study site selected was Cacra beach, a coastal ecosystem of Goa. It is a secluded beach located around 5.2 km from Bambolim, near Goa University on Taleigao Plateau. Its latitude and longitude are 15.4510° N and 73.8374° E, respectively. One can see a cluster of houses in the area with many wooden canoes and fishing nets all over. Rocky shores characterize this beachy area.

Cacra beach was selected for sampling mainly because of the huge amount of Anthropogenic activities done by the locals and tourists. Most people living around Cacra beach rely on fishing, resulting in a lot of plastic discharge in the surrounding waters. It is highly polluted with plastic nets, fishing gears, household plastic wastes, etc. It has been noticed that human activities on Cacra beach have intensified.

The 2nd Study site selected was Miramar beach, a beautiful stretch of coastline located in the state of Goa, India. It is situated at the confluence of the Arabian Sea and the Mandovi River and is known for its pristine white sands, clear blue waters, and picturesque views. It is located about 3.5 kilometres from Panaji, the capital city of Goa, and approximately 6.5 kilometres from Goa University. It is easily accessible by car or public transport and takes about 15-20 minutes to reach. Its latitude and longitude are 30.3744° N, and 86.3586° W, respectively.

The beach is approximately 2 kilometres long and is surrounded by swaying palm trees and lush greenery. It is a popular destination for tourists and locals alike, who enjoy a variety of water sports such as jet skiing, parasailing, and banana boat rides. There are several beachside shacks and restaurants where visitors can enjoy delicious Goan cuisine and refreshing drinks while taking in the stunning views of the ocean. The reason behind selecting Miramar beach as a study site is because of the high anthropogenic activities by tourists, fishermen, and local people.

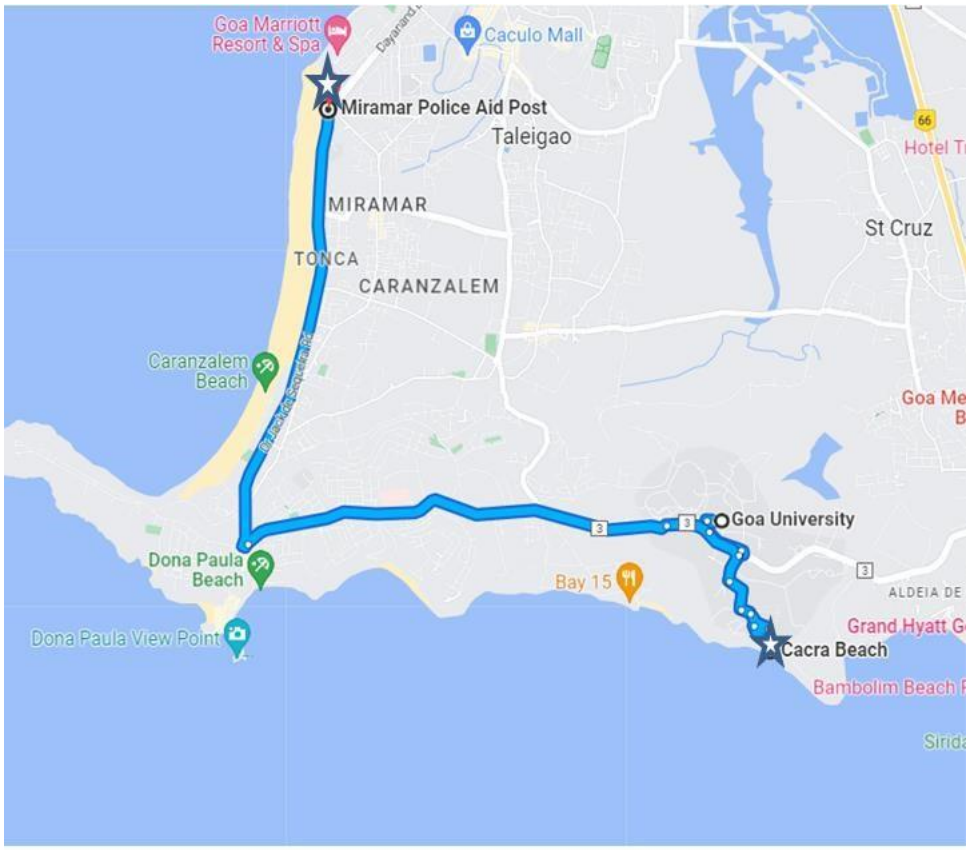

Fig 7: Study site: Cacra and Miramar (Source: Google Maps)

3.2 SAMPLING DETAILS AT CACRA BEACH:-

3.2.1 1st Sampling

On 08 February 2023, around 1.00 we went to Cacra Beach to collect Microplastic. Sampling was done on the high tide region where sandy shores are present. When we went there it was a low tide (2.06m). We collected visible plastic sample floating in water on the shores to study on plastisphere communities. Plastic samples from fore-shores was collected in ziplock bag using a sterile foreceps and brought it to laboratory for analysis.

3.2.2 2nd Sampling

On 20th February 2023, around 11:00 in the morning, we went for sampling at Miramar Beach. The sample was collected in a Zip-lock bag using sterile forceps and brought to the laboratory in an ice box for further analysis.

3.3 SUPPORTING PARAMETERS:-

- **TEMPERATURE**

PRINCIPLE: A thermometer works on the principle that solids and liquids expand when heated is known as thermal expansion. This is a fundamental property of matter, and it occurs because the atoms and molecules in a substance move more rapidly as they absorb heat energy. This increased motion causes the substance to expand. As the temperature rises, mercury expands causing it to move upwards and depict the temperature.

PROTOCOL:

- The surface water sample was collected in a bucket. From this, a mug of water was taken out and the thermometer was dipped into it.
- Readings (temperature) were noted down in (^{o}C).

- **SALINITY**

PRINCIPLE: A Refractometer is an instrument used to measure the refractive index of a substance, which is a measure of how much the substance bends light. The principle behind a Refractometer is based on the fact that light travels at different speeds in different materials. The refractive index is calculated by comparing the angle of refraction of the substance to the angle of refraction of a known standard. The difference between these two angles is used to

calculate the refractive index of the substance, which can then be read from a scale on the refractometer.

PROTOCOL:

- The water sample was collected in a bucket from the surface.The water was taken out using a dropper, 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.
- Salinity reading was noted.

3.4 LABORATORY ANALYSIS FOR ANALYSIS AND ENUMERATION OF PLASTIPHERE BACTERIA:

The collected samples were transferred to a sterile petriplate and washed with sterile saline. The plastic piece was cut into pieces using surface sterilized scissors. These pieces were then used for viable count.

3.4.1 SERIAL DILUTIONS

PRINCIPLE: Serial dilutions are a laboratory technique used to decrease the concentration of a substance in a solution. This is done by taking a known volume of the original solution and mixing it with a known volume of a solvent to create a new solution with a lower concentration. This new solution can then be further diluted using the same technique to create a series of solutions with progressively lower concentrations.

PROTOCOL:

- All used apparatus were first cleaned and autoclaved before use.
- Normal saline was prepared as per the given calculations and autoclaved.
- Then, 5 ml of normal saline was poured into a sterilized beaker (10^0).
- 4.5 ml of sterilized saline was added in (10^{-1}), (10^{-2}), and (10^{-3}) test tubes respectively.
- A small piece of plastic sample was thoroughly washed and scraped with sterile forceps in a (10^0) beaker containing normal saline.
- 0.5 ml from (10^0) to (10^{-1}) was transferred and mixed well using a pipette.
- Similarly, 0.5 ml was added from (10^{-1}) to (10^{-2}) and then mixed well.
- Dilutions were done till (10^{-3}).

3.4.2 SPREAD PLATING METHOD

PRINCIPLE: The spread plate technique is a microbiological method used to isolate and quantify bacterial colonies on solid agar media. It involves spreading a small amount of a diluted bacterial culture onto the surface of a solid agar plate using a sterile spreading tool such as a sterile glass rod or a sterile spreader.

PROTOCOL:

- All apparatus used were cleaned and autoclaved before use.
- Zobell marine agar was freshly prepared and autoclaved.
- It was poured into petriplates and kept for 24 hours to solidify.

- 0.1 ml of diluted sample from (10^{-3}) test tube was poured on the solidified ZMA plate using pipette and then spread using a flame sterilized spreader.
- Similarly, for the other two diluted samples, i.e. (10^{-1}) & (10^{-2}) same protocol was repeated.
- The plates were incubated at room temperature for 24 hours, followed by counting of the colonies observed.

3.4.3 ISOLATION OF PLASTISPHERE BACTERIA

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

STREAK PLATE METHOD

PRINCIPLE: 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.

PROTOCOL

- Master plates were then kept for incubation for 24 hours at room temperature.
- After this, colonies with characteristic colours, sizes, and forms were picked.

- They were transferred to newly prepared ZMA plates using the Streak plate method.
- Colonies were picked up using a flame sterilized loop and transferred onto the new plate. The loop was sterilized again in flame and then dragged across the plates in perpendicular directions.
- The cultures were allowed to grow for 24 hours at room temperature.

3.4.4 SUBCULTURING OF PLASTISPHERE BACTERIAL ISOLATES

PRINCIPLE: 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.

PROTOCOL:

- Isolated colonies from the previous plates were picked and transferred to another petriplate using the flame sterilized loop.
- Using the same streaking method, cultures were dragged across the plate in perpendicular direction creating a pattern of decreasing colony density and the presence of more isolated colonies.
- Purified isolates were stored on slants (ZMA) at 4˚C till further analysis.

3.4.5 CHARACTERIZATION OF PLASTISPHERE BACTERIAL ISOLATES

GRAM STAINING

PRINCIPLE: Gram staining is a differential staining technique used in microbiology to categorize bacteria based on their cell wall properties. The Gram staining principle is based on the ability of bacterial cells to retain or lose a crystal violet stain when exposed to a decolorizing agent.

PROTOCOL:

- A bacterial smear was prepared on the glass slide with a drop of saline and air dried.
- The smear was then heat fixed to the slide by passing it over the flame.
- The slide was flooded with crystal violet stain for 1 min.
- It was washed with tap water.
- Gram's Iodine was added to the smear for 1 min.
- Gram's decolourizer was used to decolourize till the blue dye stopped flowing out from the smear. (Acetone can be used as a decolourizing agent with caution, as it can decolourize the smear).
- They were again washed with tap water.
- 0.5%w/v Safranin was used as a counter stain for 20 seconds and rinsed off with water.
- The slide was allowed to air dry before examining under oil immersion objective.

3.4.6 EXPERIMENT: RESPONSE OF PLASTISPHERE BACTERIA TO DIFFERENT GROUPS OF POLLUTANTS PROTOCOL:

- All the apparatus used was first cleaned and autoclaved.
- Nutrient broth media was prepared as per the calculations and autoclaved.
- The plastisphere bacterial isolates were first revived in Nutrient broth media and kept them for 24- 48 hours.

- Revived pure cultures were then used for further analysis.

Three experiments were conducted to determine the response of plastisphere bacteria to different groups of pollutants (antibiotic – ampicillin; pesticide – monocrotophos; pharmaceutical compound - paracetamol).

1st Pollutant Experiment (Ampicillin-Antibiotic)

- All the apparatus used was first cleaned and autoclaved.
- Ampicillin was prepared of concentration 50 ug/ml.
- The Nutrient broth was also prepared for the experiment and autoclaved.
- All the cultures growth were tested against this concentration of ampicillin.
- +ve control tubes with nutrient broth and culture, -ve control tubes with nutrient broth and ampicillin (50ug/ml), and experimental tubes with nutrient broth, ampicillin, and culture in duplicate were prepared.
- Tubes were kept at room temperature for 24 hours to allow the cultures to grow.
- After 24 hours, growth was checked in all the tubes using BR BIOCHEM LIFE SCIENCES PVT. LTD colorimeter at 620nm wavelength.
- All the results were noted down.

2nd Pollutant Experiment (Monochrotophos -Pesticide)

- All the apparatus used was first cleaned and autoclaved.
- Monochrotophos was prepared of concentration 1ul/ml.
- The nutrient broth was also prepared for the experiment and autoclaved.
- All the cultures growth were tested against this concentration of monochrotophos.
- +ve control tubes with nutrient broth and culture, -ve control tubes with nutrient broth and monochrotophos (1ul/ml), and experimental tubes with nutrient broth, monochrotophos and culture in duplicate were prepared.

- Tubes were kept at room temperature for 24 hours to allow the cultures to grow.
- After 24 hours, growth was checked in all the tubes using BR BIOCHEM LIFE SCIENCES PVT. LTD colorimeter at 620nm wavelength.
- All the results were noted down.

3rd Pollutant Experiment (Paracetamol – Pharmaceutical)

- All the apparatus used was first cleaned and autoclaved.
- Paracetamol was prepared of concentration 100mg/ml.
- The nutrient broth was also prepared for the experiment and autoclaved.
- All the cultures growth were tested against this concentration of Paracetamol.
- +ve control tubes with nutrient broth and culture, -ve control tubes with nutrient broth and paracetamol (100mg/ml), and experimental tubes with nutrient broth, paracetamol and culture in duplicate were prepared.
- Tubes were kept at room temperature for 24 hours to allow the cultures to grow.
- After 24 hours, growth was checked in all the tubes using BR BIOCHEM LIFE SCIENCES PVT. LTD colorimeter at 620nm wavelength.
- All the results were noted down.

Chapter 4

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

Table 1: Selected Location Site

SR.NO.	LOCATION SITE	NO. OF BACTERIAL ISOLATES
1	Cacra	3
2	Miramar	5

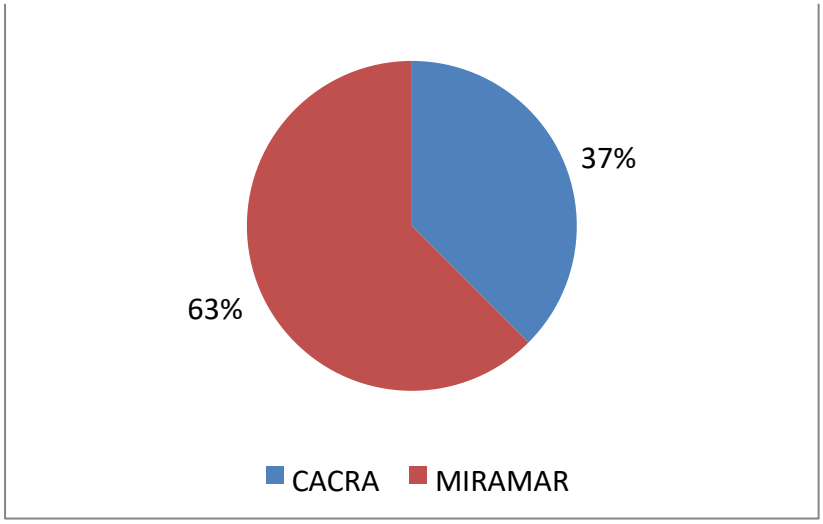

Fig 8: Proportion of Plastisphere Bacterial Isolates with respect to the different study sites.

More Bacterial isolates were found at the Miramar site than at the Cacra site (Fig.8). Temperature and salinity values were similar at both sites (Table 2). Both the study sites differed with respect to the extent of anthropogenic activity. Anthropogenic activity is higher at Miramar than at Cacra, which results in higher influx of potential plastic substrates into the water, which

could lead to large number of plastisphere communities. The Miramar site is close to Panjim city, while the Cacra site is away from the city in a secluded area.

4.1 ENVIRONMENTAL PARAMETERS

Table 2: Environmental parameters

Sr. No	Study Site	Temperature	Salinity
1.	Cacra	36.8	32
2.	Miramar	34.3	34

4.2 VIABLE COUNT

Table 3: Plastisphere bacterial isolates found at different sites and their codes.

SR. NO	CULTURE CODE	LOCATION	MEDIA
1	3	CACRA	ZMA
2	4	CACRA	ZMA
3	5	CACRA	ZMA
4	1(2)	MIRAMAR	ZMA
5	2(2)	MIRAMAR	ZMA
6	3(2)	MIRAMAR	ZMA
7	4(2)	MIRAMAR	ZMA
8	5(2)	MIRAMAR	ZMA

Viable count was below detectable levels at Cacra and 4.28×10^4 cfu/ml at Miramar.

4.3 GRAM'S STAINING RESULTS

Table 4: Characterization of Plastisphere bacterial isolates

SR. NO.	CULTURES	GRAM CHARACTER AND MORPHOLOGY	SHAPE
1	3	Gram +ve	Rods
2	4	Gram +ve	Rods
3	5	Gram +ve	Rods
4	1(2)	Gram +ve	Rods

5	2(2)	Gram +ve	Cocci
6	3(2)	Gram +ve	Cocci
7	4(2)	Gram +ve	Rods
8	5(2)	Gram +ve	Rods

All of the bacterial isolates showed Gram +ve character (Table 4). Most of the isolates showed Rod shape morphology except isolates 2(2) & 3(2) which showed cocci shape (Table 4).

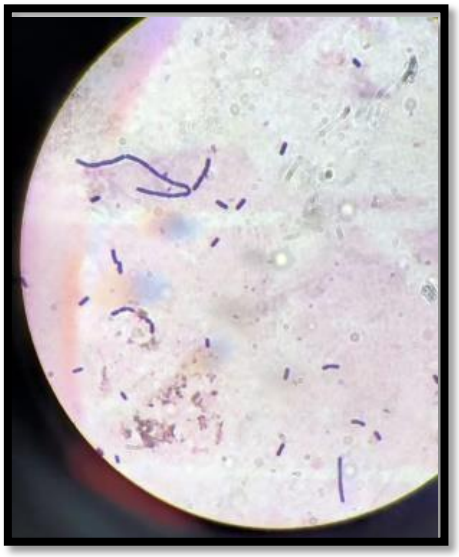

Fig 9: 3 Plastisphere bacterial isolate

4.4 GROWTH OF PLASTISPHERE BACTERIAL ISOLATES IN NUTRIENT BROTH AFTER 24 HOURS

Table 5: O.D (AT 620nm) of Plastisphere bacterial isolates growth after 24 hours in Nutrient broth media.

Sr. No	CULTURE	O.D at 620nm
1	3	0.24
2	4	0.28
3	5	0.42
4	1(2)	0.33
5	2(2)	0.22
6	3(2)	0.22
7	4(2)	0.30
8	5(2)	0.21

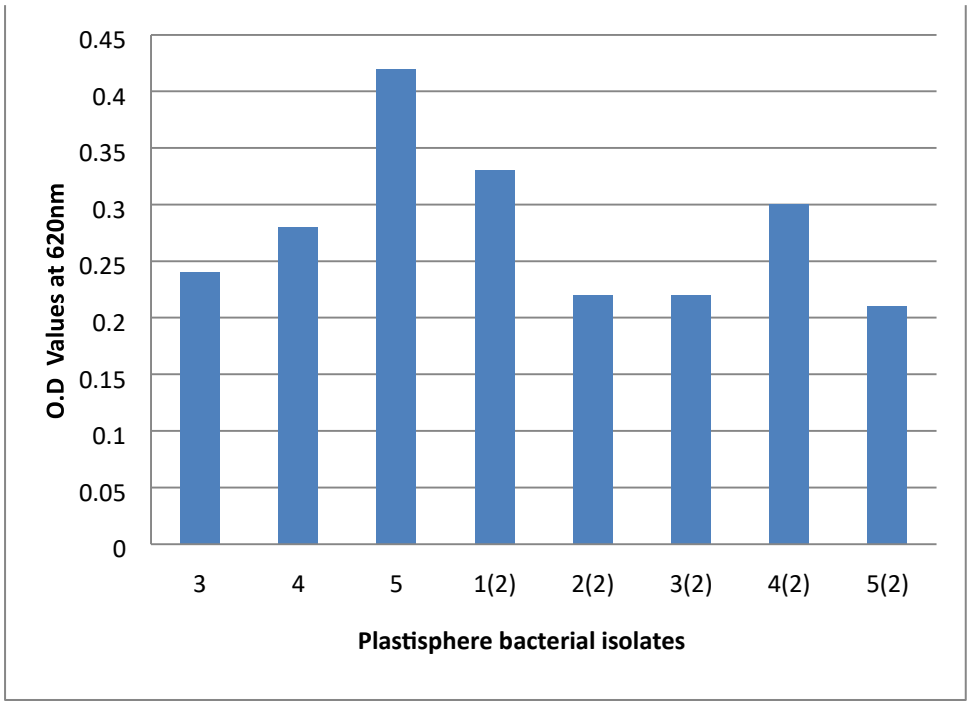

Fig 10: Plastisphere bacterial isolates growth O.D at 620nm after 24 hours

Plastisphere bacterial isolates showed abundant growth after 24 hours of incubation at room temperature in Nutrient broth, with OD at 620 nm ranging from 0.21 to 0.42 (Table 5 and

Fig.10). Isolate (5) from the Cacra study site showed highest OD compared to others, while isolate 5(2) from the Miramar study site showed the lowest OD (Fig.10).

4.5 SENSITIVITY OF PLASTISPHERE BACTERIAL ISOLATES TO DIFFERENT CLASSES OF POLLUTANTS

4.5.1 AMPICILLIN-ANTIBIOTIC

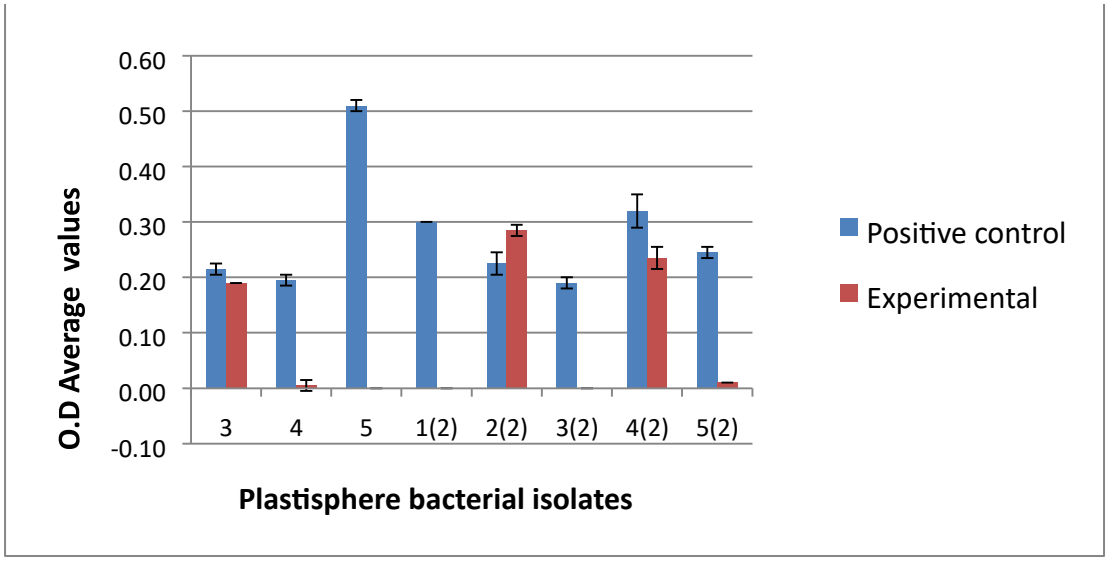

Fig 11: Plastisphere bacterial isolates growth (OD at 620 nm) in presence of 50µg/ml Ampicillin, compared to control.

Bacterial Isolates 5, 1(2) & 3(2) showed 100% inhibition by ampicillin, while the rest of the isolates, except 2(2), showed inhibition of growth. (Fig.11). Isolate 2(2) showed higher growth in the ampicillin treatment compared to control, indicating that this particular isolate is least sensitive to ampicillin (Fig.11).

4.5.2 MONOCROTOPHOS-PESTICIDE

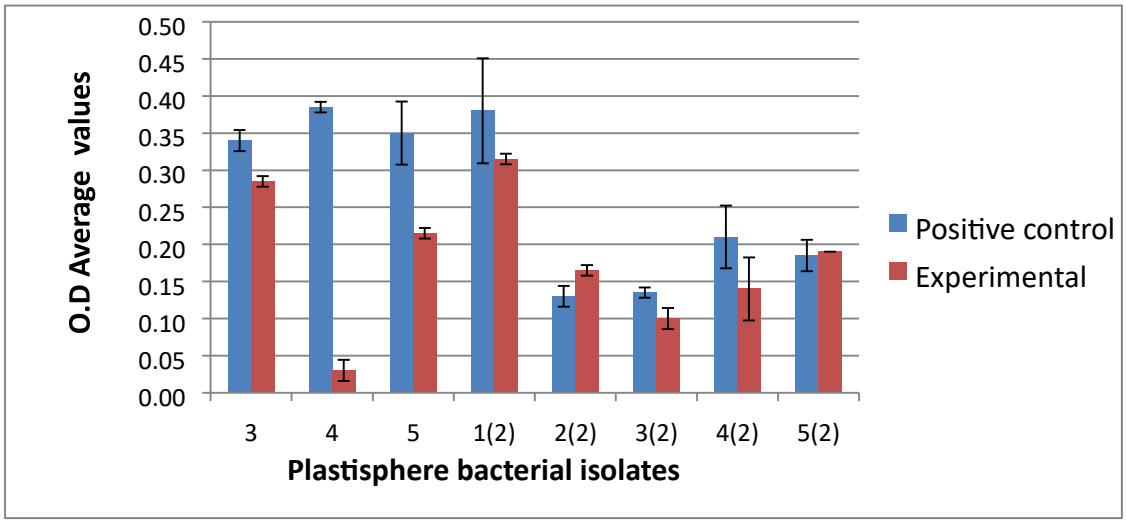

Fig 12: Plastisphere bacterial isolates growth (OD at 620nm) in presence of 1µl/ml Monocrotophos, compared to control.

All bacterial isolates, except 2(2) and 5(2) showed inhibition of growth. Isolate 4 showed the least growth in presence of monocrotophos (Fig.12). Isolates 2(2) and 5(2) showed higher growth in the monocrotophos treatment compared to control, indicating that these isolates are least sensitive to monocrotophos (Fig.12).

4.5.3 PARACETAMOL-PHARMACEUTICAL

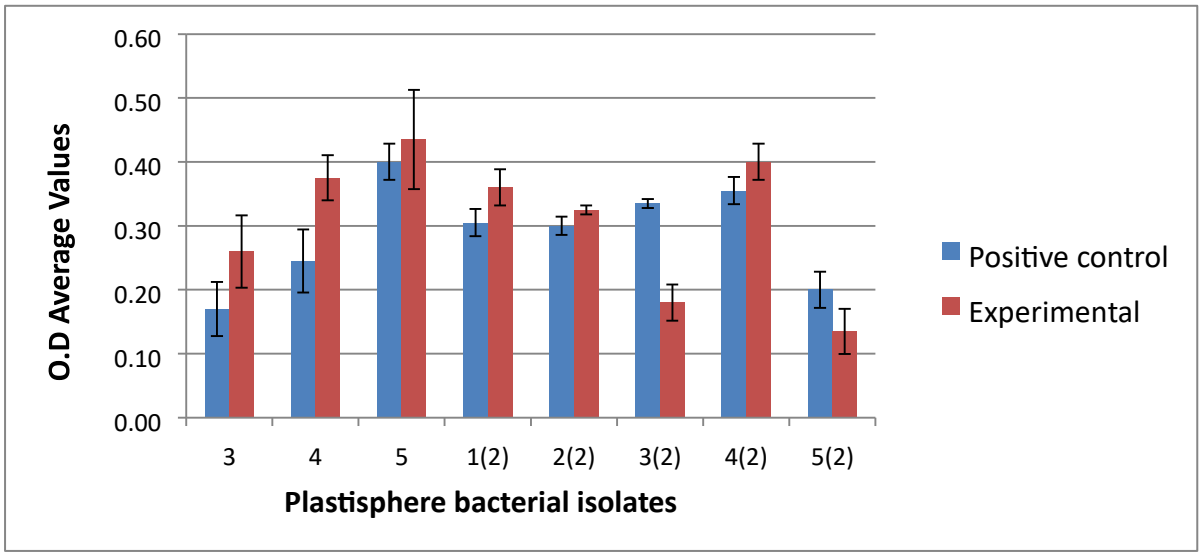

Fig 13: Plastisphere bacterial isolates growth (OD at 620nm) in presence of 100mg/ml Paracetamol, compared to control.

All bacterial isolates showed abundant growth in the presence of paracetamol, as compared to control, except isolates 3(2) and 5(2) (Fig.13). All isolates were not sensitive in the presence of paracetamol, while only 3(2) and 5(2) were sensitive toward paracetamol.

On comparing the sensitivity profile of plastisphere bacterial isolates across different pollutants, it is visible that the isolate 2(2) showed the least sensitivity toward all of the pollutants. Only in Ampicillin treatment, a few bacterial isolates [5, 1(2) & 3(2)] show 100% growth inhibition. Comparatively to other pollutants, Bacterial isolates showed the least sensitivity to paracetamol and were most sensitive toward ampicillin (Fig.11, 12 & 13). Results show that the growth of plastisphere communities is negatively impacted in the presence of antibiotic (Ampicillin). Pollutants can lead to a decrease in the diversity and abundance of microbial communities, as well as a shift in the composition of these communities. This could be due to the toxic effects of pollutants on microorganisms, which can inhibit their growth and metabolism, leading to reduction in their ability to perform ecological functions. Wang et al., (2021) reported that the introduction of pharmaceuticals into the environment has been observed to cause a reduction in the diversity of microbial communities present in the plastisphere. This may be due to the pharmaceuticals' ability to inhibit the activities of microorganisms and prevent them from adhering to the surfaces of PVC and PE. As a result, the diversity of microbial communities on PVC and PE is decreased, ultimately leading to a decrease in the stability of ecological functions. Additionally, pollutants can alter the physicochemical properties of plastic surfaces, making them less favorable for microbial attachment and growth. Overall, the presence of pollutants in the environment can have significant implications for the growth and stability of plastisphere communities, ultimately affecting the ecological functions they perform. According to the results Monocrotophos (organophosphate insecticide) presence showed inhibition to the growth of plastisphere bacterial isolates. Similar results were obtained by Garrido et al., (2019), in which Chlorpyrifos

(CPF) (organophosphorus pesticide) is commonly used to control foliage and soil-borne insect pests on a variety of food and feed crops. Microalgae exposed to CPF sorbed onto MPs reduced their growth but, for the range of CPF concentrations assayed, total inhibition was not observed. Growth inhibition of microalgae on microplastic was higher when CPF was presented dissolved. Contaminants like organochlorines, polycyclic aromatic hydrocarbons, and metals have been found to accumulate on plastic surfaces, which can cause toxicity in organisms that consume them (Rios et al., 2007; Ashton et al., 2010).

Chapter 5

SUMMARY AND CONCLUSION

5. SUMMARY AND CONCLUSION

The production of plastic objects encompasses a wide range of shapes and dimensions, including very tiny ones that measure less than 5 millimeters. These extremely small plastic pieces are commonly referred to as "microplastics" (Hortan et al.,2017). Microplastics are a growing environmental concern as they can accumulate in waterways, oceans, and soil, posing a threat to aquatic and terrestrial organisms and ecosystems (Ahmed et al., 2021; Chen et al., 2019). This study was carried out to isolate plastisphere communities and to test their sensitivity against different classes of pollutants like Antibiotic, Pesticide and Pharmaceutical. Sampling of floating plastic debris was done from two different study sites, i.e., Cacra and Miramar. Supporting parameters like water temperature and salinity were checked. After isolation and purification of eight Plastisphere bacterial isolates, their characterization was done using Gram's staining method. Most isolates turn out to be Gram +ve in nature, mostly rod shape. The sensitivity of plastisphere bacterial isolates to different groups of pollutants (Antibiotic-Ampicillin, Pesticide-Monocrotophos, PharmaceuticalParacetamol). Results showed that bacterial isolates showed the least sensitivity to paracetamol and were most sensitive toward ampicillin. Isolate 2(2) showed the least sensitivity toward all of the pollutants. From this, it was confirmed that plastisphere bacterial isolates are sensitive and react differently to different classes of pollutants. Studying the sensitivity of plastisphere communities to these pollutants can help us better understand the potential risks to human health associated with exposure to microplastics and their associated pollutants. Understanding the sensitivity of these communities to different pollutants can help us identify which pollutants may inhibit or promote plastic biodegradation and inform strategies to enhance the biodegradation of plastics in the environment.

Chapter 6
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6. References

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PLAGIARISM REPORT

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