Studies on Bacterial Pathogens Associated with Plastic Debris in Mangrove Environments

A Dissertation for

Course code and Course Title: MMI 651

Discipline Specific Dissertation

Credits: 16

Submitted in partial fulfilment of Master's Degree

M.Sc Marine Microbiology

By

MS. SAKSHI JAYANT GOVEKAR

Seat Number : 22P0390011

ABC ID: 100523084057

PRN: 201910424

Under the Supervision of

DR. PRIYA M. D'COSTA

School of Earth, Ocean and Atmospheric Sciences

Marine Microbiology

GOA UNIVERSITY

MAY 2024

Examined by:

Seal of the School

DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, "Studies on bacterial pathogens associated with plastic debris in Mangrove environments "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 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 will not be responsible for the correctness of observations / experimental or other findings given the dissertation.

I hereby authorize the University authorities to upload this dissertation on the dissertation repository or anywhere else as the UGC regulations demand and make it available to any one as needed.

Govekar

SAKSHI GOVEKAR

22PO390011

Date: 2nd May 2024

Place: Goa University

COMPLETION CERTIFICATE

This is to certify that the dissertation report, "**Studies on bacterial pathogens associated with plastic debris in Mangrove Environments**" is a bonafide work carried out by MS. SAKSHI JAYANT GOVEKAR under my supervision/mentorship in partial fulfilment of the requirements for the award of degree in the Discipline of M.Sc Marine Microbiology at the School of Earth, Ocean and Atmospheric Sciences, Goa University.

PMD

Dr. Priya M. D'Costa

Date: 02/05/24

Ghadi 31/5/24

Sr. Prof. Sanjeev C. Ghadi

Dean, School of Earth, Ocean and Atmospheric Sciences

Date:

Place: Goa University

SEOAS, GOA UNIVERSITY TALEIGAO PLATEAU GOA

School Stamp

CONTENTS

PREFACE

Mangrove ecosystems, known for their distinctive intertidal environments and diverse range of species, are essential for shielding coastlines, storing carbon, and sustaining coastal communities. Nonetheless, the increasing concern about plastic pollution poses a significant danger to the well-being and operation of these critical ecosystems. Plastic pollution in mangroves, encompassing a spectrum of plastic debris ranging from macro to microplastics, has emerged as a pressing environmental concern with far-reaching ecological and socio-economic implications.

The dissertation entitled “Studies on bacterial pathogens associated with plastic debris in Mangrove Environments” investigates the complex relationship between bacterial pathogens and plastic debris in mangrove environments, exploring how plastic debris serves as vectors in these ecosystems. The research will also improve our understanding of how plastic pollution affects the environment and the presence of bacterial pathogens on the plastic.

ACKNOWLEDGEMENT

As I stand on the threshold of completing my dissertation work entitled, “Studies on bacterial pathogens associated with plastic debris in Mangrove Environments**”.** I am overwhelmed with gratitude for the person who has been my guiding light throughout this journey. To my revered guide, Dr. Priya D’Costa. Your expertise, patience, enthusiasm, encouragement, and positive energy have been a constant source of motivation, inspiring me to strive for excellence and to push beyond my limits.

I would like to express my special thanks to our Dean Sr. Prof. Sanjeev C. Ghadi, Programme Director Dr. Varada S. Damare, teaching and non-teaching staff of the School of Earth, Ocean and Atmospheric Sciences, Laboratory Assistant Ms. Vaishali Merchant, and Mr. Rohan Marshelkar for providing me with the laboratory requirements throughout my project. I would also like to thank Ms. Imrana Shaikh for helping me.

To my dear friends and classmates, your unwavering support and encouragement have been a constant source of strength. I extend my heartfelt appreciation to my family for their endless love, and encouragement, your belief in me has been my driving force in my life.

Thank you, God, for your blessings and guidance that have led me to the successful completion of this project.

SAKSHI GOVEKAR

TABLES

FIGURES

ABBREVIATIONS USED

Entity	Abbreviation
Antibiotic Resistance Gene	ARG
Chrome Azurole S	CAS
Eosine Methylene Blue Agar	EMB
Low Density Poly Ethelene	LDPE
Low Density Polyethelene	LDPE
Luria Bertani Agar	LB
Mannitol Salt Agar	MSA
Micro Plastics	MP
Poly Ethylene	PE
Poly Propylene	PP
Posphate Buffered Saline	PBS
Scanning Electron Microscopy	SEM
Thiosulfate Citrate Bile Sats Sucrose Agar	TCBS
Vibrio cholerae Like Organisms	VCLO
Vibrio parahaemolyticus Like Organisms	VPLO
Xylose Lysine Deoxycholate Agar	XLD
Zobell Marine Agar	ZMA

ABSTRACT

Plastic pollution poses a significant threat to ecosystems worldwide, impacting marine life, terrestrial habitats, and human health. Mangroves, vital coastal ecosystems, serve as hotspots for the accumulation of plastic waste, raising concerns about their ecological and public health impacts. This study seeks to investigate the presence and attachment of pathogens living on plastic debris in mangrove environments of Chorao and Ribandar Mangroves along the coast of Mandovi River of Goa through field research and laboratory examinations. Bacteria and pathogens were tentatively identified on different media (Zobell Marine Agar, Eosin Methylene Blue (EMB) agar, Mannitol Salt Agar (MSA), Xylose Lysine Deoxycholate (XLD) agar, Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) Agar). Pathogens – *Vibrio cholerae*-Like Organisms (VCLO), *Vibrio parahaemolyticus* Like Organisms (VPLO) and *Klebsiella pneumoniae* belonging to Enterobacteriaceae family, were observed attached to plastic debris samples from these mangroves. All the isolates exhibited nil resistance to streptomycin and differential sensitivity to the rest of the antibiotics (ampicillin, chloramphenicol and nalidixic acid). Scanning Electron Microscopy (SEM) photographs revealed pit formation on plastic surfaces, corresponding to the location of the attached bacterial cells. Ultimately, findings from this dissertation will inform strategies for mitigating the environmental and public health risks associated with plastic debris contamination in coastal ecosystems.

Keywords

Plastic marine debris, plastisphere, mangroves, bacterial pathogens, *Vibrio* spp.

CHAPTER 1. INTRODUCTION

1.1 Background

Plastic pollution is a global environmental crisis that profoundly impacts ecosystems, biodiversity, and the planet's health. With the increasing production and consumption of plastic worldwide, vast quantities of plastic waste end up in the environment, posing significant threats to terrestrial and marine ecosystems.

Marine ecosystems, in particular, bear a heavy burden of plastic pollution. Research indicates that millions of metric tons of plastic enter the oceans yearly (Jambeck et al., 2015). This plastic waste poses a myriad of threats to marine biota. Sea turtles, for instance, are particularly vulnerable to ingesting plastic debris, mistaking plastic bags for jellyfish, a common prey item (Schuyler et al., 2013). Similarly, seabirds often ingest small plastic fragments, leading to physical harm and even death (Wilcox et al., 2015).

The impact of plastic pollution extends beyond individual organisms to entire marine food webs. Plastic debris can accumulate toxic chemicals from the surrounding environment, such as polychlorinated biphenyls (PCBs) and pesticides. When marine organisms ingest these plastics, they also ingest these harmful chemicals, leading to bioaccumulation and biomagnification, with potential consequences for ecosystem health and human health through seafood consumption (Rochman et al., 2013).

In addition to the direct threats posed to marine life, plastic pollution alters marine habitats and ecosystems. Coral reefs, critical marine ecosystems that support a vast array of biodiversity, are threatened by plastic debris. Plastic pollution can smother corals, block sunlight, and cause physical damage to reef structures (Lamb et al., 2018). Moreover, plastic debris can serve as a vector for invasive species, disrupting native ecosystems and biodiversity (Derraik et al., 2002).

The socio-economic implications of plastic pollution are profound, particularly for coastal communities reliant on marine resources. Plastic pollution can harm fisheries, tourism, and coastal economies, leading to economic losses and impacting livelihoods (Hoellein et al., 2014). The impacts of plastic pollution on polar ecosystems are also vast. One of the most pressing concerns is the direct threat posed to marine wildlife. Polar bears, seals, seabirds, and other iconic species often mistake plastic debris for prey or become entangled in discarded fishing gear, leading to injury, suffocation, or starvation (Wilcox et al., 2015). It also affects mangrove ecosystems, which are vulnerable, sensitive environments and support a high diversity of organisms.

Mangroves, the dynamic coastal ecosystems where land and sea converge, are highly significant environments celebrated for their robustness and rich variety of life forms. These distinct habitats, distinguished by their ability to thrive in salty conditions and complex root structures, serve as critical breeding and refuge areas for a wide array of marine and land-dwelling creatures. Mangroves fulfill a crucial function in bolstering coastal settlements, offering defense against erosion, storm surges, and tsunamis. Additionally, they act as reservoirs for carbon, contributing significantly to the absorption and storage of atmospheric carbon dioxide. (Donato et al., 2011).

Plastic pollution poses a significant threat to mangrove environments, with detrimental effects on both ecological integrity and biodiversity. The accumulation of plastic debris in mangrove ecosystems disrupts natural processes, inhibiting nutrient flow and root growth, thereby compromising the health and resilience of mangrove forests (Hossain et al., 2020). Furthermore, marine organisms inhabiting mangrove habitats are at risk of ingesting plastic particles, leading to adverse health effects such as malnutrition and internal injuries (Jahir et al., 2019). The presence of plastics also introduces toxic chemicals into the environment,

contaminating soil, water, and marine life within mangrove ecosystems (Chowdhury et al., 2020).

Bacteria and pathogens play a significant role in the degradation and transformation of plastic in marine environments, influencing its persistence and impact on marine ecosystems. As plastic debris enters the ocean, it becomes colonized by a diverse community of microorganisms, including bacteria, fungi, and algae (Keswani et al. 2016). These microbial communities, collectively known as the "plastisphere," interact with plastic surfaces and contribute to biodegradation through various mechanisms. Bacteria associated with plastic degradation produce enzymes that break down plastic polymers, such as polyethylene and polypropylene, into smaller fragments (Zettler et al., 2013). Furthermore, pathogens on plastic surfaces raise concerns regarding the spread of infectious diseases in marine organisms. Studies have documented the attachment of pathogenic bacteria, viruses, and other microorganisms to plastic debris, potentially serving as vectors for the transmission of diseases among marine species (Keswani et al., 2016). This phenomenon underscores the interconnectedness between plastic pollution and public health concerns, highlighting the need for comprehensive strategies to mitigate the impacts of plastic in marine environments.

The costs associated with cleaning up plastic debris from beaches and coastal areas place a heavy burden on local governments and communities (Cozar et al., 2014). Addressing plastic pollution requires concerted efforts at local, national, and global levels. Strategies to mitigate plastic pollution include implementing bans on single-use plastics, improving waste management infrastructure, promoting recycling and circular economy initiatives, and raising public awareness about the issue (Geyer et al., 2017).

1.2 Aim and Objectives

Aim: To investigate the prevalence of bacterial pathogens on plastic debris from mangrove environments.

Objectives:

- To isolate and characterize bacteria associated with plastic debris collected from mangroves.
- To identify and characterize bacterial pathogens present on the plastic debris.

1.3 Hypothesis

Given the widespread presence of plastic debris in mangrove environments and the known tendency of bacteria to colonize and interact with such surfaces, it is hypothesized that there exists a distinct assemblage of bacterial pathogens associated with plastic debris in mangrove ecosystems.

1.4 Scope

The results of this work will provide interesting insights into the bacterial pathogens associated with plastic marine debris, particularly whether plastic in mangroves can be considered as a reservoir for bacterial pathogens of human interest.

CHAPTER 2. LITERATURE REVIEW

2.1 COLONIZATION ON PLASTICS

The high hydrophobicity of plastics produces a strong interface when these materials are put in water, hindering microbial attachment. Nevertheless, these surfaces are rapidly covered by organic matter, collectively referred to as the eco corona, which decreases the hydrophobicity of the surfaces and facilitates microbial colonization (Galloway et al., 2017).

2.2 PLASTISPHERE

Microplastics provide a substrate and unique niche for various microbial cultures to attach and colonize, forming biofilms in the environment. Such biofilms are termed as plastisphere, consisting of a complex community, including bacterial, archaeal, eukaryotic microorganisms and microscopic animals, forming a new mini-ecosystem. Microscopic (phenotypic) and molecular sequencing (genotypic) data complemented in providing evidence for microbial phototrophy, symbiosis, heterotrophy (including phagotrophy), and predation in the analyses of Polypropylene (PP) and Polyethylene (PE) samples. SEM photomicrographs revealed a rich eukaryotic and bacterial microbiota inhabiting both PP and PE samples. Cell counts from random images identified over 50 distinct morphotypes covering between 0 and 8% of the plastic surface area. Particularly intriguing were round cells approximately 2 μm in diameter found embedded in pits on the PMD surface. Often arranged in rows or patches, these pits closely matched the shape of the contained cells, which included dividing cells indicating active growth. Although these cells were not identified, they represented the third most common morphotype observed, following diatoms and filaments. DNA sequencing analyses confirmed that the communities were consistently distinct between plastics and the surrounding seawater. For instance, filamentous cyanobacteria with photosynthetic capabilities, such as *Phormidium* and *Rivularia* OTUs, were present on plastics but absent from seawater samples dominated by unicellular *Prochlorococcus.* SEM photomicrographs also revealed the presence of

cyanobacteria resembling *Plectonema*-like genera. Diatoms, belonging to various Bacillariophyta genera including *Navicula, Nitzschia, Sellaphora, Stauroneis*, and *Chaetoceros,* were prominently observed on plastics, known for their association with substrates and biofilm formation in aquatic environments. Additionally, other protists with known photosynthetic representatives, such as prasinophytes, rhodophytes, cryptophytes, haptophytes, dinoflagellates, chlorarachniophytes, chrysophytes, pelagophytes, and phaeophyta, were identified through DNA sequence data analysis (Zettler et al., 2013).

The initial attachment of microorganisms to plastic surfaces is affected by the wettability of the material, with hydrophilic surfaces (water contact angle below 90°) generally promoting faster biofilm formation compared to hydrophobic surfaces (water contact angle above 90°) (Wright et al., 2017). The physicochemical properties of polymeric media, such as polypropylene, polyethylene, polystyrene, polyurethane, high-density polyethylene, and polyvinyl chloride, are widely used in biological wastewater treatment plants due to their excellent mechanical strength, lightweight, chemical inertness, and durability. These materials significantly affect biomass adhesion, and their surface hydrophobicity/hydrophilicity is a critical factor for bacterial adhesion. Hydrophilic carriers typically have a higher energy surface than hydrophobic carriers, implying that bacteria attach and develop more easily on biofilm carriers with hydrophilic surfaces. However, the relationship between wettability and bacterial adhesion is still debated, with some studies finding no consistent correlation between the two. The use of waste PET plastic bottles as fixed biofilm carriers in wastewater treatment has been studied, showing removal efficiency of organic pollutants between 70 and 90%, meeting the discharge standard of Indonesian domestic wastewater. This approach is effective in reducing environmental pollution, particularly in developing countries with limited resources for waste treatment (Wright et al., 2017).

2.3 FACTORS INVOLVED IN FRAMING THE PLASTISPHERE

2.3.1 Water

Plastics in marine environments face challenges when stranded on shorelines, limiting life development above the tidal line. Microbes in the Plastisphere exhibit remarkable adaptation to seawater salinity, with potential variations as riverine plastics transition into marine conditions. While some microbes can withstand temporary exposure to seawater's 3% salt content, sustained growth and survival in marine settings necessitate significant evolutionary adjustments (Simon et al., 2017).

2.3.2 Nutrients

Sunlit oceans are highly oligotrophic, i.e., low in nutrients. Oceans transition from mesotrophic in coastal areas to ultraoligotrophic in subtropical gyres. As currents transport plastics, they converge in all five oceanic gyres, known as plastic hotspots or garbage patches, a significant proportion of marine plastic debris is therefore exposed to ultraoligotrophic conditions (Bryant et al., 2016). While planktonic organisms have evolved fascinating mechanisms to overcome such deprivation of nutrients, these may be dispensable for microbes within biofilms. The intimate closeness of microbes within biofilms increases the number of interactions as well as the entrapment of nutrients obtained from their oligotrophic surroundings. Thus, while planktonic organisms living in immensely dilute systems rely on distant and transient microbe−microbe exchange of nutrients, the build-up and cycling of nutrients between phototrophic and heterotrophic microorganisms within biofilms is much more effective. It is therefore not surprising to find that Plastispheres are more productive than their surrounding planktonic counterpart communities. As a proof of concept, they showed that plastic exposed to recirculating oligotrophic seawater was able to develop a considerable biofilm sustained by phototrophic organisms and build up a substantial amount of organic carbon and nitrogen, the latter by impoverishing the surrounding water (Bryant et al., 2016).

2.3.3 Energy

The energy source driving the Plastisphere's activity is influenced by the plastic's location within the water column. Plastics segregate based on their density, with buoyant plastics in sunlit ocean layers being colonized by photosynthetic primary producers. These producers generate labile photosynthate, a carbon and energy source that fuels the Plastisphere's growth. In these well-lit regions, large biofilms thrive on this energy source, outcompeting potential biodegrading organisms. Conversely, denser plastics sinking to darker, light-deprived layers force the Plastisphere to derive energy from degrading compounds in the environment, the biofilm itself, or the plastic additives they are colonizing, leading to a reduction in the Plastisphere's size (Bryant et al., 2016).

2.4 SIGNIFICANCE OF PLASTISPHERE MICROORGANISMS IN BIODEGRADATION OF PLASTIC POLYMERS

Microorganisms that were capable of degrading polymers have been studied and isolated from the natural environment. Polymer materials used for microbial degradation, such as polyethylene and polypropylene, were investigated. The microbial species associated with polymer degradation, including *Streptococcus*, *Klebsiella*, *Micrococcus*, *Staphylococcus,* and *Pseudomonas*, were identified. The biodegradability of polyethylene was improved by blending it with various additives, leading to enhanced auto-oxidation and reduction in molecular weight, making it easier for microorganisms to degrade these lower molecular weight polymers (Zeenat et al., 2021).

Bacillus vallismortis bt-dsce01 successfully degraded LDPE by 75% after 120 days of incubation (Skariyachan et al., 2017). *Aspergillus oryzae* strain A5 and *B. cereus* strain A5 degraded LDPE by 36.4% and 35.72%, respectively, after 112 days of incubation. *B. siamensis*, was capable of degrading 8.46% of LDPE after 90 days of incubation. When polyethylene undergoes thermal-photo oxidation, it produces various compounds like ketones, aldehydes, carboxylic acids, alkanes, alcohols, lactones, dicarboxylic acids, and esters. Despite these

efforts, the microbial degradation of PE has remained slow. The high molecular weight of PE has restricted its utility as a substrate for many enzymatic reactions. In the biodegradation process of PE, two crucial reactions occur a decrease in molecular weight and oxidation (Skariyachan et al., 2017).

2.5 PLASTISPHERE AS A VECTOR FOR PATHOGENS

Increasing studies of plastisphere have raised public concern about microplastics (MPs) as vectors for pathogens, especially in aquatic environments. However, the extent to which pathogens affected human health through MPs remained unclear, as controversies persisted regarding the distinct pathogen colonization on MPs as well as the transmission routes and infection probability of MP-associated pathogens from water to humans. In a review, it was critically discussed whether and how pathogens approached humans via MPs, shedding light on the potential health risks involved. Drawing on cutting-edge multidisciplinary research, it was shown that some MPs may have facilitated the growth and long-range transmission of specific pathogens in aquatic environments, ultimately increasing the risk of infection in humans. MP- and pathogen-rich settings, such as wastewater treatment plants, aquaculture farms, and swimming pools, were identified as possible sites for human exposure to MP-associated pathogens. The review emphasized the need for further research and targeted interventions to better understand and mitigate the potential health risks associated with MP-mediated pathogen transmission (Zhong et al., 2023).

Plastics have been found to harbor a distinct microbiome containing potential pathogens and antibiotic-resistance genes (ARGs) (Zadjelovicet al., 2023). This microbiome, known as the plastisphere, differs from the surrounding water microbiome and resembles biofilms found on wood surfaces. Notably, opportunistic pathogens like *Pseudomonas aeruginosa*, *Acinetobacter*, and *Aeromonas*, along with various ARG subtypes, were prevalent on all surface-related microbiomes, particularly on weathered plastics. In contrast, a different set of

potential pathogens such as *Escherichia, Salmonella, Klebsiella*, and *Streptococcus*, along with distinct ARGs, dominated in the planktonic compartment. Through genome-centric analysis, 215 Metagenome Assembled Genomes (MAGs) were assembled, linking ARGs and virulence-related genes to their host. One MAG belonging to *Escherichia*, which was abundant in water, exhibited a higher number of ARGs and virulence factors compared to other MAGs, highlighting the potential virulence of these pathogenic groups. Furthermore, incubations with environmentally relevant antibiotic concentrations led to an increase in the prevalence of corresponding ARGs, with different riverine compartments, including plastispheres, responding differently to each antibiotic as studied by (Zadjelovicet al., 2023).

Isolated systems, such as oceanic islands, have increasingly encountered significant issues related to microplastic debris on their beaches. (Sanchez et al., 2023) The formation of microbial biofilm on the surface of microplastics found in marine environments provided potential habitats for microorganisms to survive within the biofilm. Furthermore, microplastics had served as a vehicle for the dispersal of pathogenic organisms, establishing a new route of exposure for humans. In this study, the microbial content (FIO and *Vibrio spp*. and *Staphylococcus aureus*) of microplastics (fragments and pellets) collected from seven beaches of the oceanic island of Tenerife, in the Canary Islands (Spain), had been determined. The results indicated that *Escherichia coli* had been present in 57.1% of the fragments and 28.5% of the pellets examined. Regarding intestinal Enterococci, 85.7% of the fragments and 57.1% of the pellets had tested positive for this parameter. Lastly, 100% of the fragments and 42.8% of the pellets analyzed from the various beaches contained *Vibrio spp.* This study demonstrated that microplastics had acted as reservoirs of microorganisms that could enhance the presence of bacteria, indicating fecal and pathogenic contamination in bathing areas (Sanchez et al., 2023).

Heterotrophic bacteria in seawater samples were predominantly dominated by Pelagibacter and other free-living picoplanktonic bacterial groups but exhibited distinct abundance patterns in the plastic samples (Bowley et al.2020). A notable observation was the prevalence of a member of the genus *Vibrio*, constituting nearly 24% of the polypropylene sample. This was remarkable as *Vibrio* members typically account for less than 1% of the community, with *V. harveyi* being a rare exception. *Vibrios* are recognized for their rapid growth rates, which could explain their occasional dominance in the Plastisphere. The *Vibrio* sequence found in high abundance on the polypropylene plastic sample was related to the type species of *V. natriegens*, a known nitrogen fixer. However, this sequence also shared 100% identity with various nontype strain vibrios, including *V. harveyi, V. alginolyticus, V. owensii, V. azureus, V. parahaemolyticus, V. campbellii, V. diabolicus, V. communis, and V. rotiferianus*, all recent additions to GenBank (Bowley et al., 2020).

Pathogenic microorganisms that had been identified based on the typical colony morphology on selective media were as follows: purple-colored colonies with a green metallic sheen were noted as *E. coli* on EMB agar; green colonies with a black center as *Salmonella* spp. on Hektoen Enteric agar; grey greenish colonies as *Listeria* spp. on PALCAM; and yellow and green colonies as *Vibrio* spp. on TCBS. The mean abundance and total counts per milliliter were tabulated. Monitoring fecal pollution in water had been conducted by enumerating coliforms to predict the presence of pathogens, with significant wastewater contamination leading to higher numbers of indicator bacteria in water bodies. In this study, common pathogenic bacteria such as *E. coli, Listeria, Salmonella*, and *Vibrio* spp. were enumerated, and the counts of fecal indicators and human pathogenic bacteria were found to be $3.9 \text{ X } 10^3$ cfu/ml (Poharkar et al., 2016).

Sampling had been carried out for two seasons (pre-monsoon and post-monsoon) in 2011 and 2012. A total of 60 (40%) out of 150 samples had been suspected positive for

Salmonella spp. (Poharkar et al., 2016) A similar study was reported by Grisi et al. (2010) in an industrially affected mangrove habitat from Paraiba do Norte River (Brazil), which had a 25% occurrence of *Salmonella* spp. Out of the 60 isolates, 22 (36.6%) had been from the pre-monsoon season and 38 (63.3%) had been from the post-monsoon sampling, implying their prevalence throughout the year. The occurrence of *Salmonella* had been influenced by environmental parameters such as temperature, rainfall, and salinity. *Salmonella* counts had been higher in the post-monsoon season, while temperature and salinity values had been higher in the pre-monsoon season. The upwelling and turbulence of water and seepage and runoff from the land and estuaries during the monsoon season may have contributed to an increase in *Salmonella* spp., corresponding with the high numbers obtained in the present study. An increase in the frequency of *Salmonella* spp. has been noted with an increase in wastewater pollution in water (Poharkar et al., 2016).

A study by Silva et al. (2019) showed that among the 14 plastic debris samples analyzed using the ATR-FTIR technique, 35.71% (5/14) were classified as Polyethylene (PE), 28.57% (4/14) as Polypropylene (PP), and 35.71% (5/14) as Teftalene polyethylene (PET). Soft plastic samples, except for SP1-SFB, were characterized as PE (SP1-MR, SP2-MR, SP1-PB, SP2-PB, and SP2-SFB), while hard plastic samples were identified as PP (HP1-EPA, HP2-PB, HP1-SFB) and PET (HP2-EPA, HP1-MR, HP2-MR, HP1-PB, and HP2-SFB). A total of 120 bacterial strains were isolated from water and plastic samples, with 59 presumptively identified as *E. coli* (44 with virulence genes confirmed by PCR) and 61 identified biochemically as *Vibrio* spp., with 59 confirmed as *Vibrio* spp. by PCR. *E. coli* strains showed virulence genes like *EaeA, iaL, Eagg, stx1, stx2, st,* and *lt* corresponding to different virulence serovars. Fifty-nine *Vibrio* strains had the rRNA 16s gene, with 12 identified as species including *V. mimicus, V. vulnificus* (Silva et al., 2019).

2.6 PREVALENCE OF ANTIBIOTIC RESISTANCE GENES IN PLASTISPHERE BACTERIA

Samples were analyzed using Raman spectra to confirm the polymer type, showing a sample similarity of polystyrene (PS), polypropylene (PP), polyethylene (PE), polyethylene terephthalate (PET), and polyvinyl chloride (PVC) of more than 80%. (Sun et al., 2023). The abundance of plastic waste in different mangrove areas varied, with 2.49 items/m^2 in living areas, 2.04 items/m^2 in aquaculture pond areas, and 3.44 items/m^2 in protected areas. Notably, mangroves in aquaculture pond areas contained less plastic waste due to regular cleaning by humans, while protected area mangroves had a higher abundance of plastic waste, particularly PS plastic. This phenomenon is attributed to PS entering protected area mangroves through tidal flow and being trapped by the dense roots, as PS easily breaks into small particles. Additionally, the plastic abundance on beaches in the Central Caribbean Coast was 4.54 items/m^2, and on the continental coast of Chile, it was 27 items/m^2, exceeding that found in typical mangrove areas. Among them, the percentages of ARGs corresponding to macrolides, tetracycline, fluoroquinolones, penicillanic, and phenylacetone were in the ranges of 14.26–14.94%, 12.75–13.73%, 8.93–9.95%, 6.94–7.43%, and 5.02–5.75%, respectively. The ARGs were categorized into 36 based on antibiotic families, with 33 being common to the three mangroves studied. A total of 175 ARGs were identified on plastic wastes in the three mangroves, representing 91.11% of the total ARGs detected. Proteobacteria and Bacteroidetes were the dominant microbial communities on plastic wastes in the three typical mangroves, accounting for 69.13–80.00% of the total microbial communities. *Vibrio,* a pathogenic bacterium within Proteobacteria, represented 2.31% of the total bacterial genera in the aquaculture pond area mangrove. These findings highlighted the significant concern regarding the high abundance of pathogenic bacteria in these environments. The presence of multiple microbes was strongly correlated with ARGs, indicating that a single microbe could carry multiple ARGs enhancing antibiotic resistance. Microbes were identified as potential hosts for most antibiotic-resistance

genes, suggesting that microbes could facilitate the transmission of these genes. The combination of antibiotic resistance genes and plastics posed a higher risk of transmission in mangroves, emphasizing the critical need for improved plastic waste management to prevent the spread of antibiotic resistance genes by reducing plastic pollution in these ecosystems (Sun et al., 2023).

CHAPTER 3. METHODOLOGY

3.1 STUDY AREAS

3.1.1 Ribandar Mangroves

Figure 3.1: Location of Ribandar Mangrove Sampling Site

The 1st sampling site selected was mangroves along the Ribandar coast (Fig. 3.1). Ribandar town is situated on the banks of the Mandovi River, offering picturesque views and a serene atmosphere. Its latitude and longitude are 15.5013° N and 73.8666° E, respectively. It is 10 km away from Goa University Taleigao Plateau.

The Ribandar mangrove site was chosen for sampling primarily due to the significant anthropogenic activities carried out by the residents and tourists. Substantial discharge of plastic from the surroundings has led to the site being polluted to a great extent. Ribandar mangroves are polluted with fishing nets, household/hotel plastic waste, and so on (Fig. 3.2).

Figure 3.2: Polluted Ribandar Mangrove site.

3.1.2 Chorao Mangroves

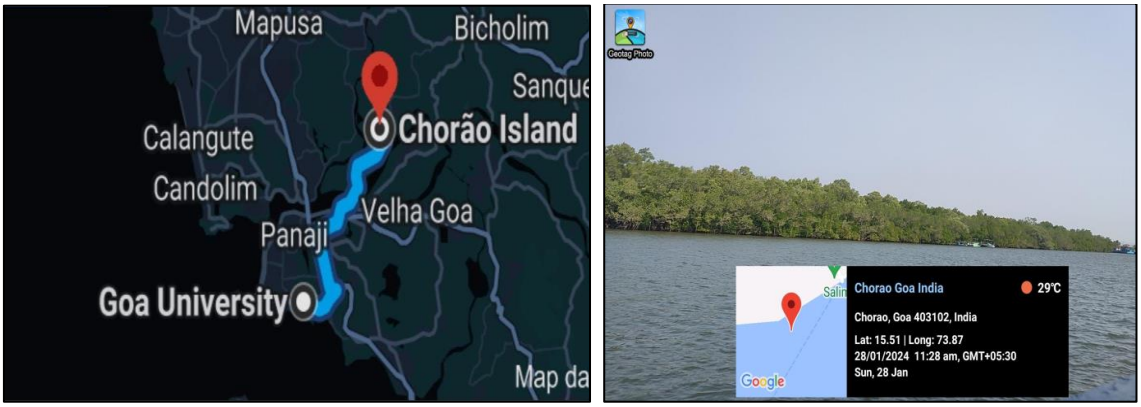

Figure 3.3: Location of Chorao Mangrove Sampling Site**.**

The 2nd sampling site selected for study was the mangroves of Chorao (Fig. 3.3). Chorao, also called Choddnnem or Chodan, is a big island on the Mandovi River near Tiswadi, Goa, India. It's the largest among the 17 islands in Goa. The total area of Chorao Island is 423.75 hacters which has a mangrove cover of about 250 hacters. The Chorao Mangroves in Goa are located at approximately 15.4960° N latitudes and longitude 73.8794° E. It is 11 km away from Goa University Taleigao Plateau.

This site was chosen for sampling because Chorao mangroves are one of the protected sites. The Chorao mangroves in Goa are a significant ecological feature known for their rich biodiversity and environmental importance. These mangroves are often visited by tourists and nature enthusiasts for their natural beauty and unique ecosystem. Conservation efforts are underway to protect the Chorao mangroves and their associated ecosystems, recognizing their ecological importance and the need to preserve them for future generations. But pollution is still a concern.

Figure 3.4: Polluted Chorao Mangrove site.

3.2 COLLECTION OF PLASTIC SAMPLES

Plastic samples were collected using sterile forceps and gloves. Each sample was cut into approximately 20cm by 15cm size pieces. Then, the sample was gently washed 2-3 times with autoclaved, filtered seawater to ensure that the plastic was free of sediment and other unwanted particles. After washing, the sample was wrapped in aluminum foil, put into an ice box, and brought to the laboratory for further analysis.

Sampling of plastic samples was carried out in Ribandar mangroves on 13 January 2024 at around 10:30 am during low tide. Details of samples are provided in (Figure:3.5) Environmental parameters like salinity, temperature, and pH were also studied.

Figure 3.5: Details of plastic samples collected from Ribandar mangroves.

Sampling of plastic samples was carried out in Chorao mangroves on 28 January 2024 at around 10:30 am during low tide. Details of samples are provided in Fig. 3.5. Parameters like salinity, temperature and pH were also studied.

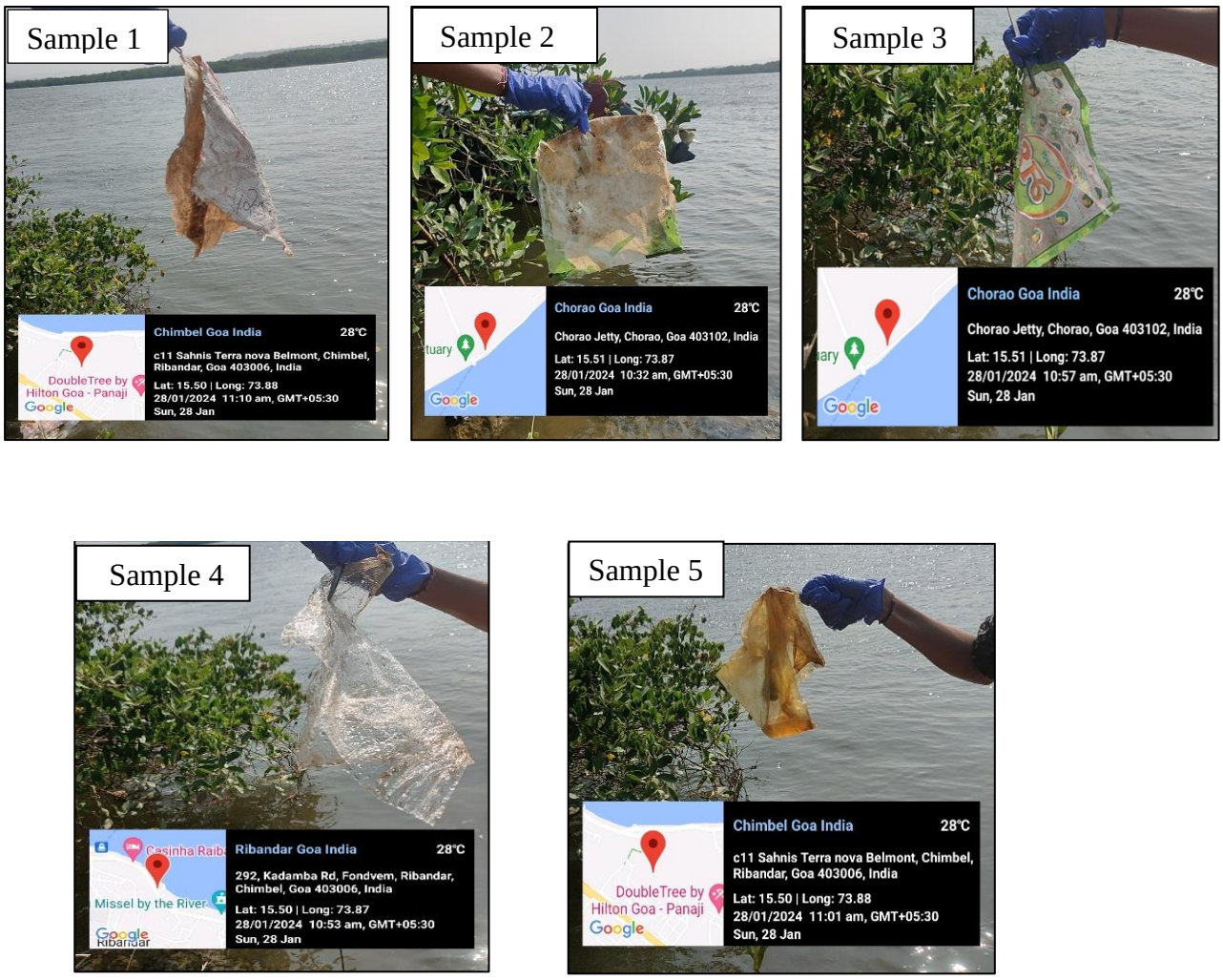

Figure 3.6: Details of plastic samples collected from Chorao mangroves.

3.3 ENVIRONMENTAL PARAMETERS

Temperature, salinity and pH of the mangrove water samples were measured.

Temperature: The surface mangrove water sample was collected in a plastic beaker. A thermometer was dipped into the water in the beaker without touching the sides or the bottom of the beaker and readings were noted down in (°C).

Salinity: The surface mangrove water sample was collected in a plastic beaker. First 2-3 drops of distilled water were put on the refractometer with the help of a dropper and the lid was covered ensuring no air bubbles are formed. The refractometer showed 0 readings after which the refractometer was wiped with tissue paper. Then 2-3 drops of mangrove water sample were put on the refractometer with the help of a dropper and the lid was covered ensuring no air bubbles were formed. The reading was noted down. Again, the sample was drained and the refractometer was wiped with tissue paper rinsed with distilled water, and cleaned with tissue paper.

pH: The surface mangrove water sample was collected in a plastic beaker. Then, pH paper was dipped into the sample and color change on the pH paper was compared with the pH chart. pH reading of the water sample was noted down.

3.4 LABORATORY ANALYSIS

3.4.1 Isolation of bacteria and pathogens on different media

After bringing the samples to the laboratory, the following procedure was followed. Under sterile conditions, the aluminum foil containing samples was opened with the help of surface-sterilized forceps and cut into 5 small pieces of size approximately 1.5cm by 1.5cm using surface-sterilized scissors. Each sample was washed 5-6 times with autoclaved seawater, dried, and carefully placed in the center of different agar medium plates. (Zobell Marine Agar, Eosin Methylene Blue (EMB) agar, Mannitol Salt Agar (MSA), Xylose Lysine Deoxycholate (XLD) agar, Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) Agar. Two control plates of each media were maintained. Open control: Agar medium plates were opened in sterile conditions for 10-15 seconds. Closed control: Closed Agar plates of each media were kept as closed control. The plates were then wrapped using newspaper and incubated. ZMA Plates were incubated at Room Temperature for 24/48hrs. EMB, MSA, XLD, and TCBS plates were incubated at 37°C for 24/48hrs, following which growth was checked for.

3.4.2 Isolation and Subculturing of Bacteria and Pathogens Using Streak Plate Method

a. Streak Plate Method

The streak plate method is a microbiological technique utilized to separate and purify bacterial colonies from a mixed culture. This method involves using a sterile inoculating loop to streak a small portion of the mixed culture onto an agar plate in parallel lines. The loop is then sterilized and drawn across the agar surface perpendicularly, spreading the bacteria thinly. This process is repeated multiple times, leading to a gradual decrease in bacterial density on the plate. By spreading the bacteria thinly and systematically, they are compelled to grow in distinct colonies, facilitating the identification and isolation of individual bacterial colonies. These isolated colonies can be further analyzed for various microbiological studies.

The plates inoculated with plastic samples were checked for bacterial growth at the end of the incubation period. Colonies with characteristic colors, sizes, and forms were picked, transferred to newly prepared ZMA plates using the Streak plate method, and isolated using the streak plate method. The bacterial cultures were allowed to grow for 24 hours at room temperature and pathogens were allowed to grow at 37°C for 24 hours.

Colonies that appeared to be pure were subjected to three cycles of streaking, to ensure purity of the cultures, and finally maintained on ZMA slants at 4^{o}C.

3.4.3 Characterization of bacterial isolates

a. Crystal Violet Assay for Attachment of Isolates On 24well Micro Titer Plates

The procedure by Naik et al. (2018) was followed for the crystal violet assay. Crystal violet assay involves staining adherent cells that are attached to cell culture plates. This assay relies on the detachment of adherent cells from the culture plates during cell death. Live cells remain attached and are stained with Crystal Violet, a dye that binds to ribose-type molecules like DNA in nuclei. After staining, dead detached cells are washed away, and the remaining

attached live cells are measured by absorbance at 600 nm. The amount of Crystal Violet staining is directly proportional to the cell biomass attached to the plate, allowing for the inference of cell viability and cytotoxicity levels. The microtiter plates are made up of polystyrene which is a type of plastic. So here attachment of isolates is checked.

A sterile 24 microtiter plate was taken, to which 500µl of ZMB was added using a micropipette. Then 500µl of 24-hour-old culture maintained in ZMB was added to the broth. 1000µl of ZMB was put in one well as a control for the experiment. The plates were incubated at Room temperature (ZMA) and 37°C (rest of the media for pathogens) for 24hrs. After the incubation period, the plates were drained and washed with autoclaved phosphate-buffered saline (PBS). Later, the plates were washed twice with autoclaved distilled water. Plates were then dried for 15-30 minutes on tissue paper. 1000µl of 1% Crystal Violet solution was added to each well and kept for 45 minutes at room temperature. Excess Crystal Violet dye was washed off by rinsing with sterile distilled water 2-3 times. The plates were dried. 2000µl of 30% acetic acid was added to each well. O. D of each culture was taken at 600 nm by taking 30% acetic acid as blank. Fifteen cultures showing maximum O.D. were selected for carrying out further experiments.

b. Antibiotic Resistance Using Disc Diffusion Method

In this method, antibiotic-impregnated disks are placed on agar plates previously inoculated with bacterial suspensions. The antibiotics then diffuse radially outward through the agar, creating a concentration gradient. After an incubation period, inhibition zones form around the disks, indicating the susceptibility of the bacteria to the antibiotics. The diameter of these inhibition zones is measured and interpreted according to established clinical breakpoints to determine the effectiveness of the antibiotics against the tested bacteria.

Sterile ZMA plates were prepared. The 24-hour culture was spread plated thoroughly on ZMA plates using a surface-sterilized spreader. With the help of forceps, four different

antibiotics (Ampicillin 10mcg, Chloramphenicol 30mcg, Nalidixic acid 30mcg, Streptomycin10mcg) discs were placed in each of the four quadrants marked on the plates. Bacterial cultures were incubated at Room Temperature and pathogens at 37°C. After the incubation period, the zone of inhibition around the antibiotic disc was measured using a ruler and results were noted down in centimeters.

c. Gram staining

A clean grease-free slide was taken. A smear of loopful 24-hour culture was made in the middle of the slide. The slide was heat-fixed by passing it through the blue flame. The slide was covered with a crystal violet solution and left to stain for about 1 minute. Then the slide was rinsed with distilled water to remove excess stain. Gram's iodine solution was added to the slide and left for about 1 minute. The slide was rinsed again with distilled water to remove excess iodine. The slide was decolorized with alcohol. Then the slide was rinsed with distilled water to remove any remaining decolorizer. The slide was counterstained with safranin for about 1 minute. The slide was rinsed with distilled water and blotted dry. The slide was examined at 100X under an oil immersion lens.

d. Siderophore Production Assay

The procedure by Arora et al. (2017) was followed for the determination of siderophore production. CAS agar plates were prepared by mixing 20ml CAS reagent in a 180ml sterilized LB medium. Four 24-hour-old cultures were spot-inoculated on each plate. After inoculation, the plates were incubated for 24-48 hours at RT and 37°C respectively, and observed for the formation of yellow-orange zones around the spot-inoculated cultures.

3.5 Adherence of bacterial isolates to plastic using Scanning Electron Microscopy (SEM)

Five cultures were chosen for this analysis. Aliquots of 5000 µl ZMB were added to each of the 5 test tubes. Approximately 1cm by 1cm autoclaved Low-Density Poly Ethelene

(LDPE) was added using sterile forceps. Aliquots (400ul) of 24-hour-old cultures were inoculated in 5 test tubes. The test tubes were incubated at 37°C for 48 hours. Plastic samples were picked up from ZMB, washed with sterile seawater, and fixed in 4% paraformaldehyde for 2-23 hours. Subsequently, they were transferred to 50% ethanol in phosphate-buffered saline (PBS) and stored at -20°C, till analysis. The plastic samples were dehydrated on ice through an ethanol series of 50%, 70%, 85%, and 95% (10 mins in each concentration) followed by 3 cycles (15 mins each) in 100% ethanol; air dried, sputter coated with gold and observed with a Scanning Electron Microscope.

CHAPTER 4. ANALYSIS AND CONCLUSIONS

4.1 ANALYSIS

4.1.1 Environmental Parameters

Parameters like temperature and pH at both sites were almost similar except for salinity i.e. in Ribandar mangroves it was 32 and in Chorao mangroves it was 29 (Table 4.1).

Table 4.1: Environmental parameters of both Mangrove sampling site

PARAMETERS	RIBANDAR MANGROVES	CHORAO MANGROVES
Salinity	32	29
Temperature	26˚C	25˚C
pH	7	7

4.1.2 Isolation of Bacteria and Pathogens on Different Media

No growth was observed in open and closed control plates indicating that there was no media contamination during the inoculation of plastic samples. Growth was observed on two out of five ZMA media plates. Growth was observed in all five TCBS media plates. Growth on MSA was observed on two out of five samples and EMB media plates did not show any growth after 24 hours of incubation. (Table 4.2). Increased growth was observed on ZMA, MSA, XLD and EMB media plates after 48 hours; of incubation (Fig. 4.1) (Table 4.3).

Table 4.2: Growth after 24 hours of incubation of plastic debris sample from Ribandar Mangroves.

Media	Open control	Closed control	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
ZMA	-	-	+	-	+	-	-
TCBS	-	-	+	+	+	+	+
MSA	-	-	+	+	-	-	-
XLD	-	-	-	-	-	-	-
EMB	-	-	-	-	-	-	-

Table 4.3: Growth after 48 hours of incubation of plastic debris sample from Ribandar Mangroves.

Media	Open control	Closed control	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
ZMA	-	-	+	+	+	+	-
TCBS	-	-	+	+	+	+	+
MSA	-	-	+	+	+	+	-
XLD	-	-	+	+	+	-	-
EMB	-	-	+	+	+	-	-

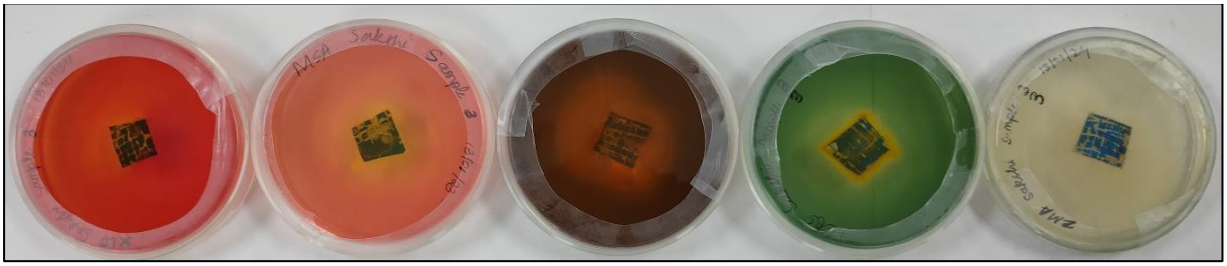

Figure 4.1: XLD, MSA, EMB, TCBS and ZMA media plates with growth of bacteria and pathogens around the plastic sample 3 from Ribandar mangroves.

Growth was observed on most of the ZMA, XLD, and TCBS media plate sample plates. Growth was observed on only one sample on the MSA media plate. Whereas there was no single growth on EMB media plates after 24 hours of incubation (Table 4.4; Fig. 4.2). Increased growth was observed on MSA and EMB media plates after 48 hours of incubation.

Table 4.4: Growth After 24 Hours of Incubation of plastic debris sample from Chorao Mangroves.

Media	Open control	Closed control	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
ZMA	-	-	+	+	+	-	+
TCBS	-	-	+	-	+	+	+
MSA	-	-	+	-	-	-	-
XLD	-	-	+	+	-	-	+
EMB	-	-	-	-	-	-	-

Table 4.5: Growth After 48 Hours of Incubation of plastic debris sample from Chorao Mangroves.

Media	Open control	Closed control	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
ZMA	-	-	+	+	+	+	+
TCBS	-	-	+	+	+	+	+
MSA	-	-	+	+	+	+	-
XLD	-	-	+	+	+	-	+
EMB	-	-	+	-	+	-	-

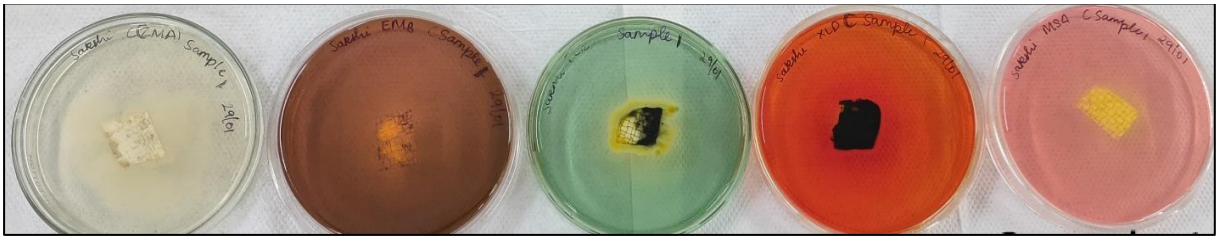

Figure 4.2: ZMA, EMB, TCBS, XLD and MSA media plates with the growth of bacteria and pathogens around the plastic sample 3 from Chorao mangroves.

A total of 39 bacterial isolates were obtained on ZMA, TCBS, MSA, XLD and EMB media on plastic samples collected from Ribandar mangroves, and 34 bacterial isolates were obtained on plastic samples collected from Chorao mangroves (Table 4.6). Pathogens, *Vibrio cholerae*-Like Organisms (VCLO), *Vibrio parahaemolyticus*-Like Organisms (VPLO) and *Klebsiella pneumoniae* were present on samples from Ribandar and Chorao mangroves (Table 4.7). VCLO were observed on S1, S3, And S5. VPLO were observed on S1, S2, and S5, there were no pathogens observed on S4 of Ribandar mangroves. VCLO were also observed on S1 and S4. VPLO were observed on S1 and S5 and there were no pathogens obtained on S3 of Chorao mangroves (Table 4.8).

Table 4.6: No. of Bacterial Isolates on different media.

Media	Number Of Bacterial Isolates	
	RIBANDAR	CHORAO
ZMA	10	12
TCBS	6	7
MSA	9	7
XLD	8	6
EMB	6	2
TOTAL	**39**	**34**

Table 4.7: Pathogens observed on plastic debris samples of both sites.

Pathogens	RIBANDAR	CHORAO
Staphylococcus aureus	-	-
Escherichia coli	-	-
Shigella spp.	-	-
Salmonella spp.	-	-
Vibrio cholerae-Like Organisms (VCLO)	+	+
Vibrio parahaemolyticus-Like Organisms (VPLO)	+	+
Klebsiella pneumoniae	+	+

Table 4.8: Sample-wise distribution of pathogens obtained on plastic samples**.**

	RIBANDAR		CHORAO	
Sample no.	VCLO	VPLO	VCLO	VPLO
S1	+	+	+	-
S2	-	+	-	+
S3	+	-	-	-
S4	-	-	+	-
S5	+	+	-	+

Many isolates belonging to the family Enterobacteriaceae were recovered on EMB and XLD media. *Enterobacter aerogenes* were observed on S2, S3, S4 and S5. *Proteus mirabilis* were observed on S1, S2 and S5. *Klebsiella pneumoniae* was observed on S3 and S5. *Escherichia coli* was only observed on S1. *Enterobacter cloacae* was only obtained on S5 of Ribandar mangroves (Table 4.9). *Enterobacter aerogenes* were observed on S1, S2, S3, and S5. *Proteus mirabilis* were observed on S1, S2, S3 and S4. *Escherichia coli* was only observed

on S1. *Enterobacter cloacae* and *Klebsiella pneumoniae* were not observed on any of the samples of Chorao mangroves (Table 4.10).

Table 4.9: Sample-wise distribution of bacteria belonging to Enterobacteriaceae obtained on plastic samples from Ribandar**.**

Bacteria	Sample no.				
	S1	S2	S3	S4	S5
Enterobacter aerogenes	-	+	+	+	+
Proteus mirabilis	+	+	-	+	-
Klebsiella pneumoniae	-	-	+	-	+
Escherichia coli	+	-	-	-	-
Enterobacter cloacae	-	-	-	-	+

Table 4.10: Sample-wise distribution of bacteria belonging to Enterobacteriaceae obtained on plastic samples from Chorao**.**

Bacteria	Sample no.				
	S1	S2	S3	S4	S5
Enterobacter aerogenes	+	+	+	-	+
Proteus mirabilis	+	+	+	+	-
Klebsiella pneumoniae	-	-	-	-	-
Escherichia coli	+	+	-	-	+
Enterobacter cloacae	-	-	-	-	-

4.1.3 Crystal Violet Assay for Attachment of Isolates On 24well Micro Titer Plates

A total number of 73 bacterial isolates were tested for crystal violet assay, out of which 44 of bacterial isolates showed O.D. greater than 1(not mentioned in the table 4.11). From

which 27 bacterial isolates were from Ribandar Mangroves and 17 were from Chorao Mangroves (Table 4.11).

Table 4.11:O. D. readings of Crystal Violet Assay.

CHORAO MANGROVES		RIBANDAR MANGROVES	
Bacterial isolates	O. D	Bacterial isolates	O. D
Control	0.3291	Control	0.2014
CS1Z1	0.8520	RS3Z1	0.7295
CS3Z3	0.8984	RS2T1	0.4026
CS1X1a	0.7863	RS5M1a	0.3442
CS5T1	0.9606	RS5E2	0.8920
CS2X1	0.8869	RS2E2a	0.9433
CS3E2	0.5685	RS2X2	0.6289
CS3E1	0.8446	RS2M2	0.9979
CS3X1	0.6445	RS2M3	0.7860
CS5T2	0.7156	RS3X2	0.6259
CS4T1	0.8223	RS3E2	0.7484
CS2X2	0.8436	RS5X2	0.3955
CS3M2	0.7715	RS2E2b	0.9249
		RS4T2	0.7030
		RS3T2	0.9772
		RS5X1	0.6111
		RS3E1	0.4757
		RS4X1	0.7810

*The rest of the isolates showed O.D greater than 1**.**

4.1.4 Characterization of plastic-associated bacterial isolates.

A total of 15 isolates showing high OD values were selected for further characterization – antibiotic resistance, Gram staining and siderophore production.

4.1.4 *a Antibiotic Resistance Using Disc Diffusion Method.*

Among all the plastic-associated cultures from Ribandar mangroves, only RS5T1 showed resistance to Ampicillin. Whereas culture RS1M2, RS2T2, RS1Z1 and RS2Z2a were not resistant to all the four tested Antibiotics.RS2T2 culture showed highest resistance to Ampicillin and Nalidixic acid and Streptomycin and RS5T1 showed highest resistance to chloramphenicol (Figure 4.3-4.5).

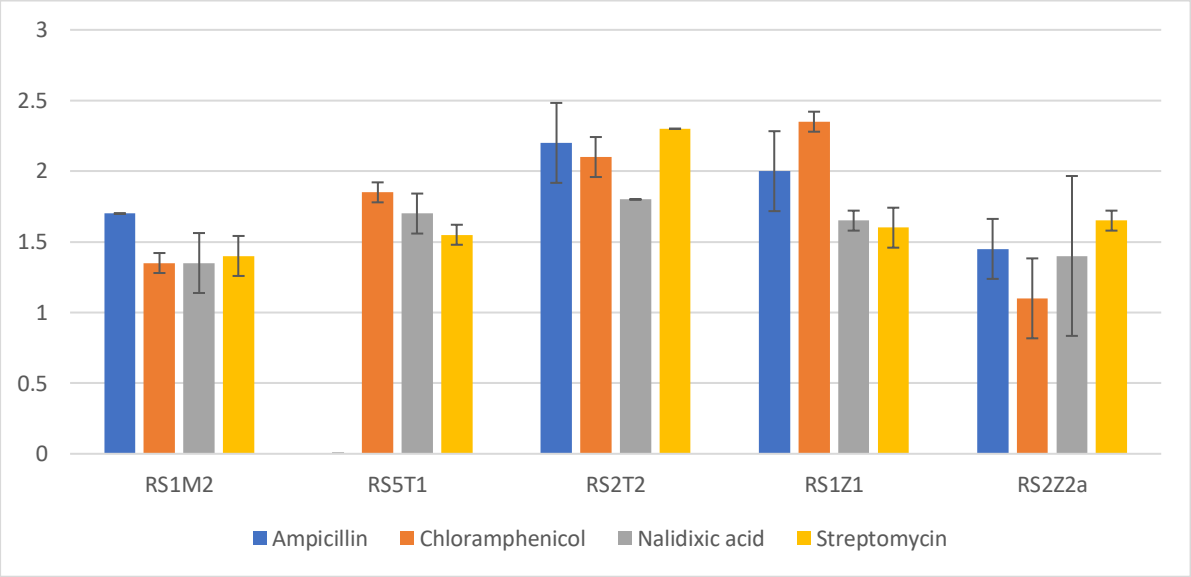

Figure 4.3: Antibiotic Resistance of Ribandar mangroves bacterial isolates

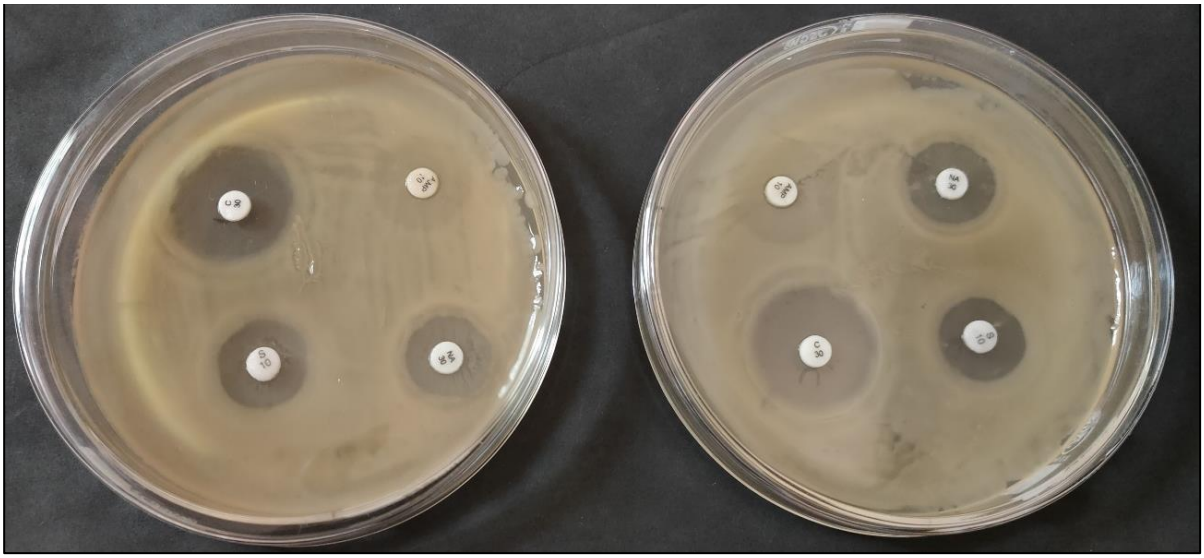

Figure 4.4: Nil resistance of RS1Z1 culture against the four tested Antibiotics (shown in duplicates).

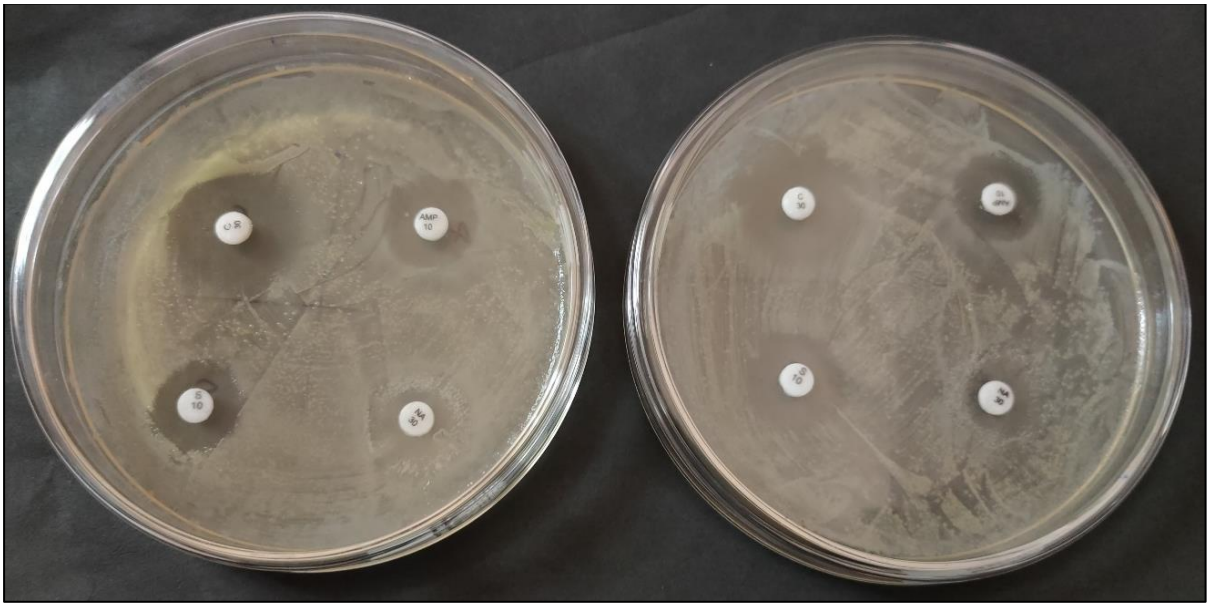

Figure 4.5: Nil resistance of RS1M2 culture against the four tested Antibiotics (shown in duplicates).

Considering the plastic-associated bacterial isolates from Chorao mangroves, 5 cultures showed sensitivity to all 4 Antibiotics. No single culture was resistant to all four antibiotics. Culture CS4Z2, CS5Z2, CS4Z3, CS5Z3 were resistant to Ampicillin. Culture CS4Z2 and

CS4Z3 were resistant to Chloramphenicol. Culture CS1M1 and CS4Z3 were resistant to nalidixic acid. None of the cultures were resistant to streptomycin (Figure 4.6-4.8).

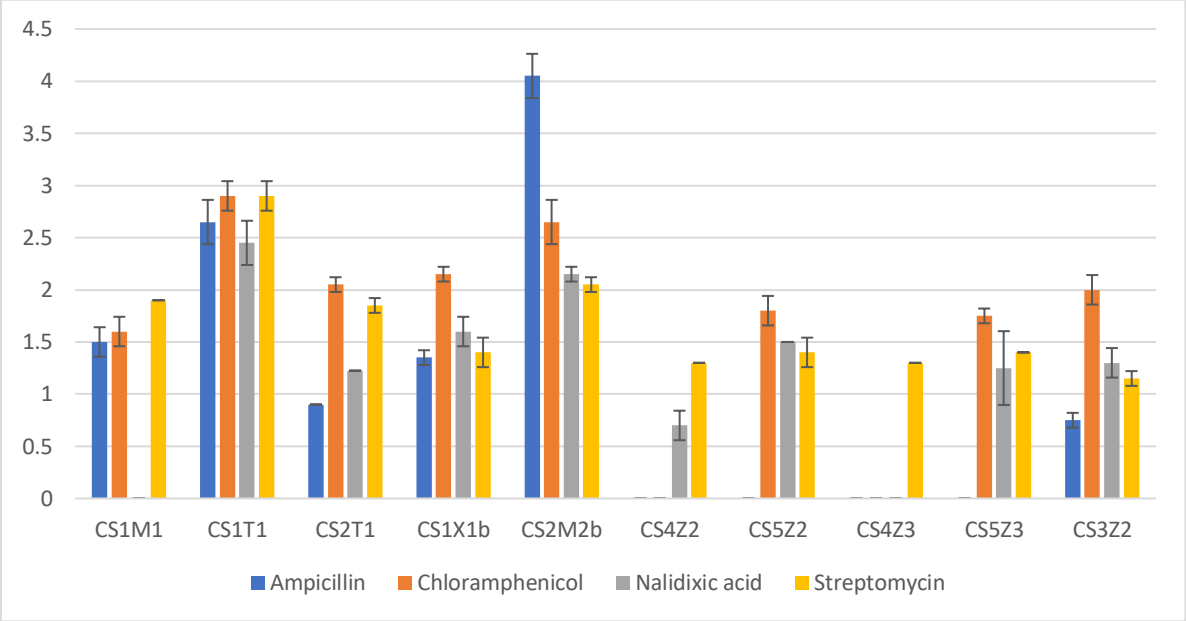

Figure 4.6: Antibiotic Resistance of Chorao mangroves bacterial isolates.

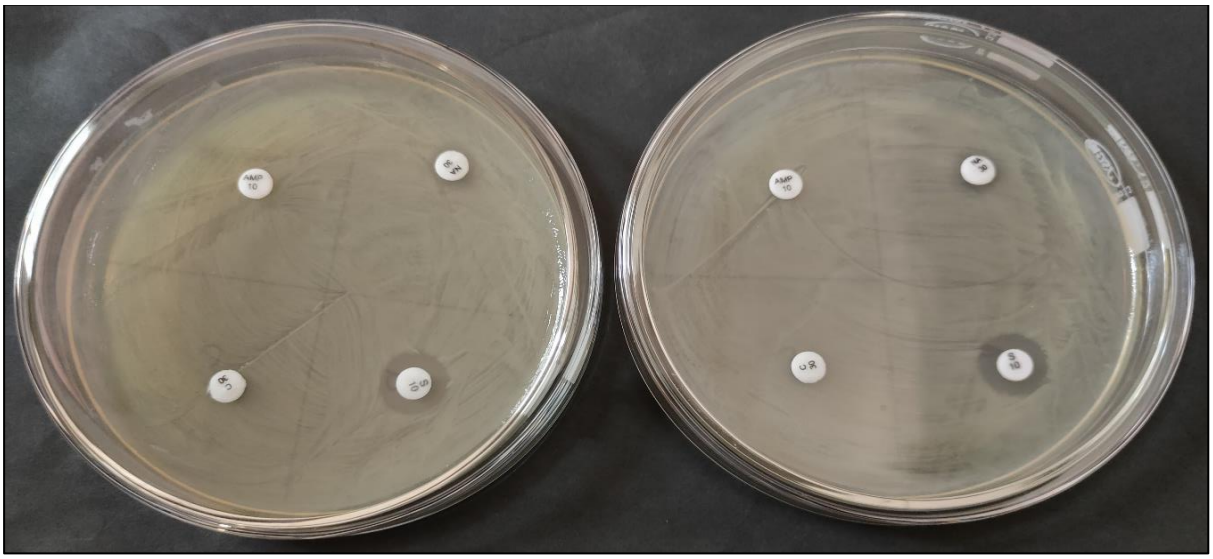

Figure 4.7: CS4Z2 culture showing resistance to Ampicillin and Chloramphenicol (no zones of inhibition) and sensitivity to Streptomycin and Nalidixic acid (zones of inhibition). The two plates represent duplicates maintained for each antibiotic.

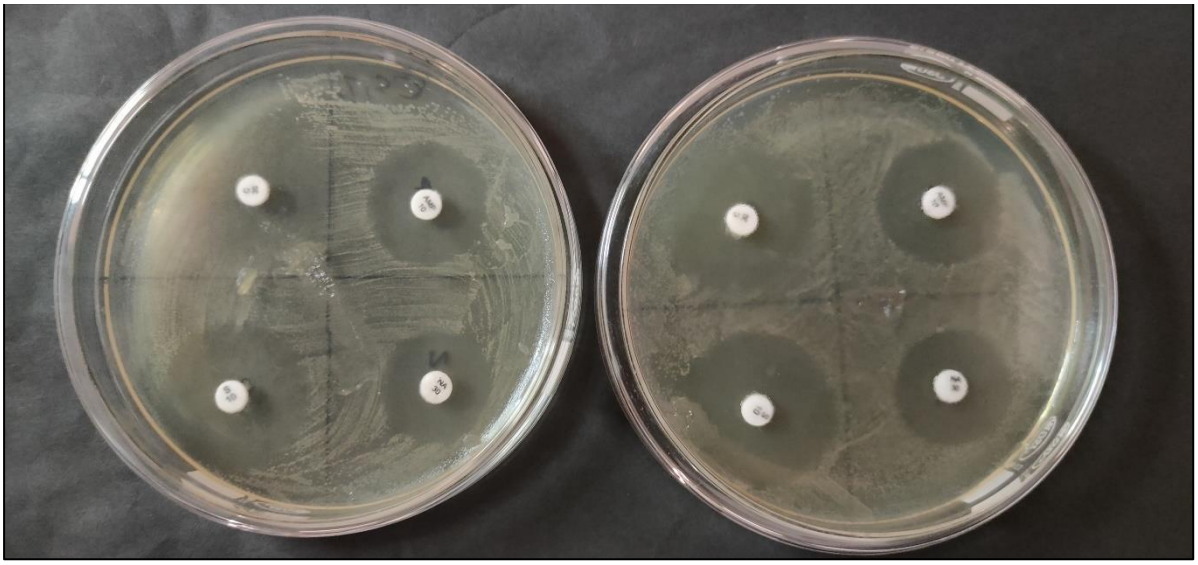

Figure 4.8: Nil resistance of CS1T1 culture against the four tested Antibiotics (shown in duplicates).

4.1.4 b *Gram staining and siderophore production*

Out of fifteen cultures, five cultures were Gram-negative coccobacilli, one culture was found to be Gram-negative bacilli, one culture was found to be Gram-positive bacilli two cultures were found to be Gram-positive cocci, four were found to be Gram-negative rods and rest were Gram-positive rods. And ten cultures were found to be positive for the Siderophore production assay and the rest five were negative (Tables 4.12-4.13; Figure 4.9).

Table 4.12: Gram nature and Siderophore production of five bacterial isolates Ribandar mangroves

	GRAM STAINING	SIDEROPHORE PRODUCTION
RS1M2	Gram -ve coccobacilli	-
RS5T1	Gram -ve coccobacilli	+
RS2T2	Gram -ve coccobacilli	+
RS1Z1	Gram -ve bacilli	+
RS2Z2a	Gram +ve cocci	-

Table 4.13: Gram nature and Siderophore production of 10 bacterial isolates from Chorao mangroves.

	GRAM STAINING	SIDEROPHORE PRODUCTION
CS1M1	Gram +ve bacilli	+
CS1T1	Gram -ve rods	+
CS2T1	Gram -ve coccobacilli	-
CS1X1b	Gram -ve rods	+
CS2M2b	Gram -ve coccobacilli	+
CS4Z2	Gram +ve rods	-
CS5Z2	Gram -ve rods	+
CS4Z3	Gram +ve rods	+
CS5Z3	Gram -ve rods	+
CS3Z2	Gram +ve cocci	-

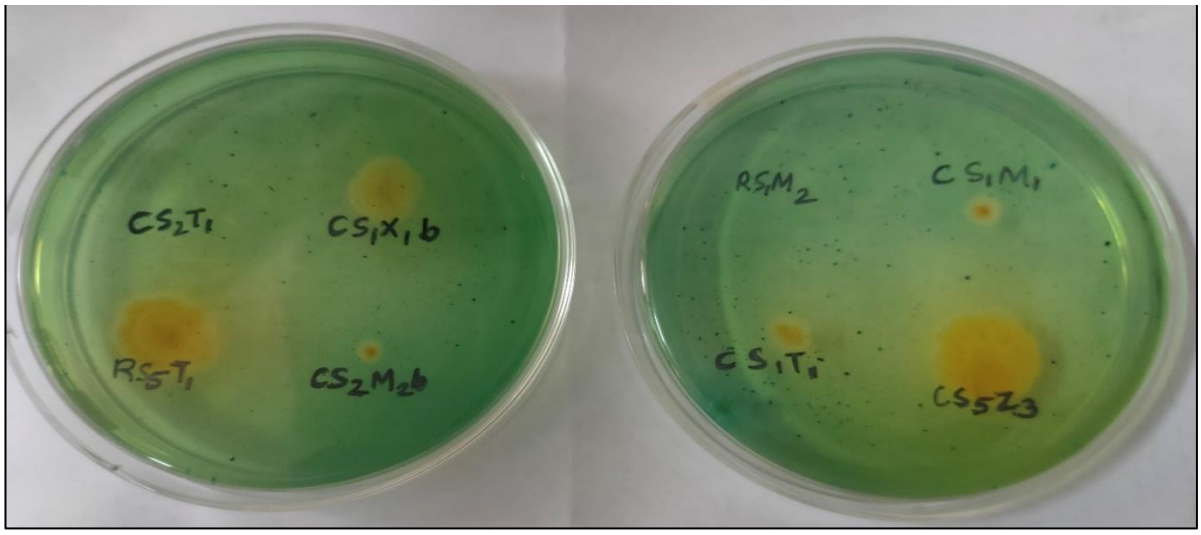

Figure 4.9: CS1X1b, RS5T1, CS2M2b, CS1T1, CS5Z3 and CS1M1 culture showing yellow orange zone around bacterial cultures indicating siderophore production.

4.1.5 Adherence of bacterial isolates to plastic using Scanning Electron Microscopy (SEM)

SEM images gave a morphological view of the bacterial isolates and their attachment to Low Density Polyethylene (LDPE). Pit-like depressions around cultures CS1X1b, CS1T1, RS1M2 and RS2T2 (Figures 4.10-4.15). Interestingly RS1M2 showed unusual morphological characteristic (Figure 4.12-Figure 4.14) when growing on LDPE polymer.

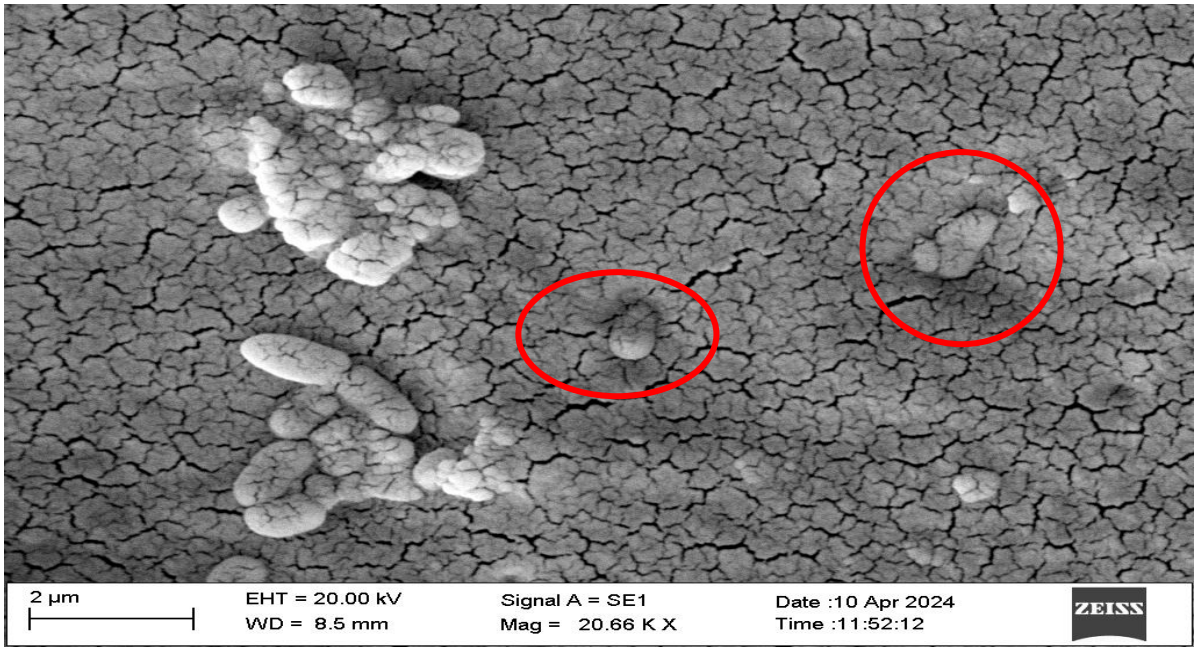

Figure 4.10: SEM photograph of CS1X1b culture showing adherence to plastic. Red circle indicates depression in plastic surface where the bacterial cell has attached.

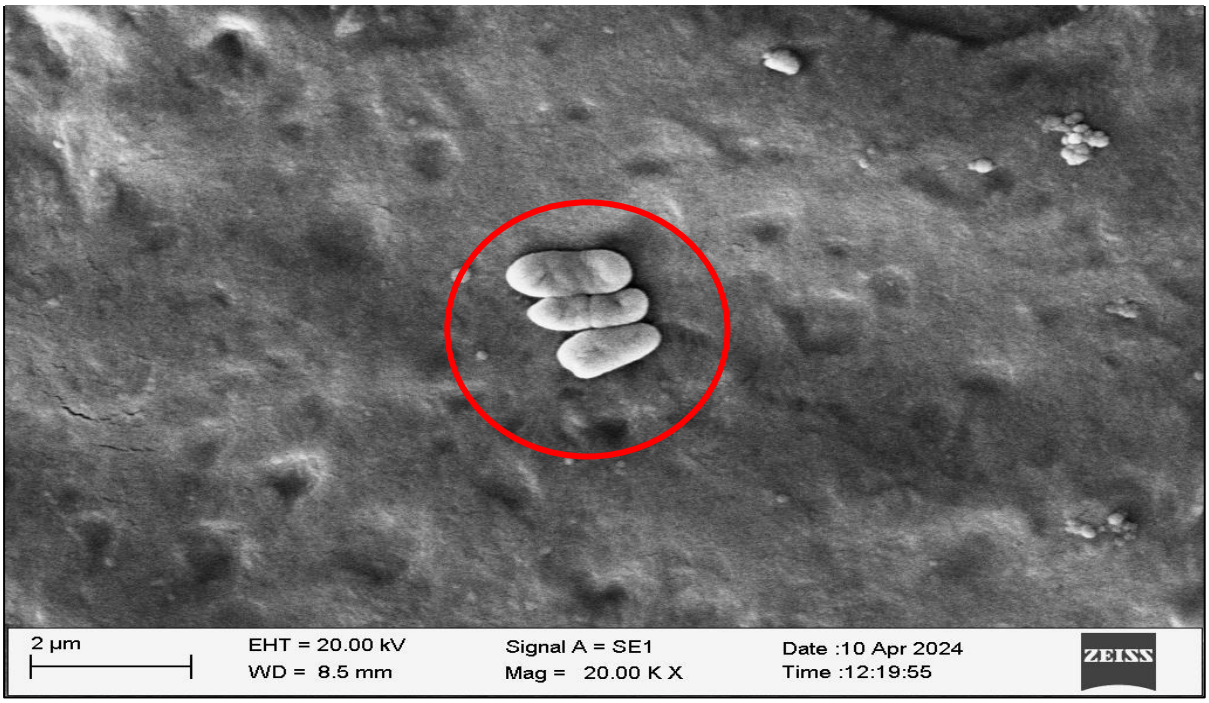

Figure 4.11: SEM photograph of CS1T1 culture showing adherence to plastic.

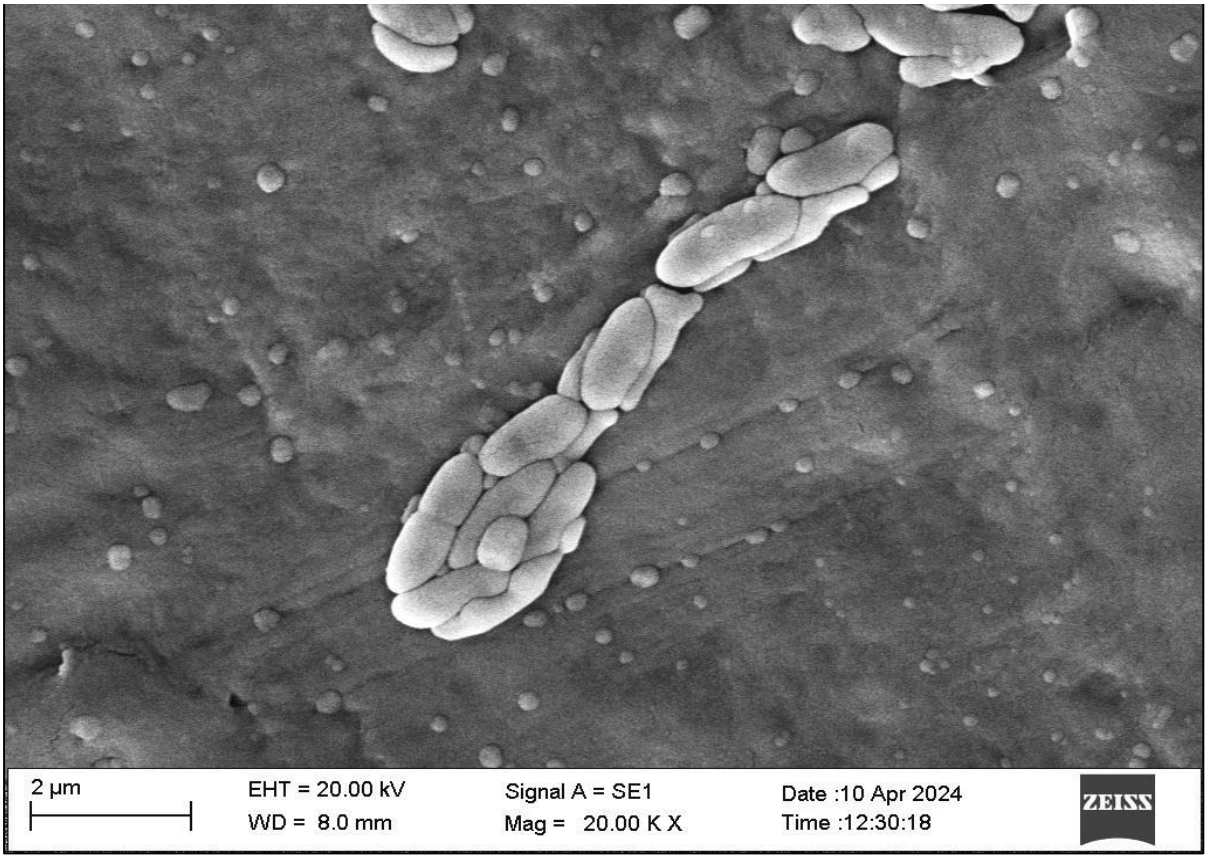

Figure 4.12: SEM photograph of RS1M2 culture showing adherence to plastic.

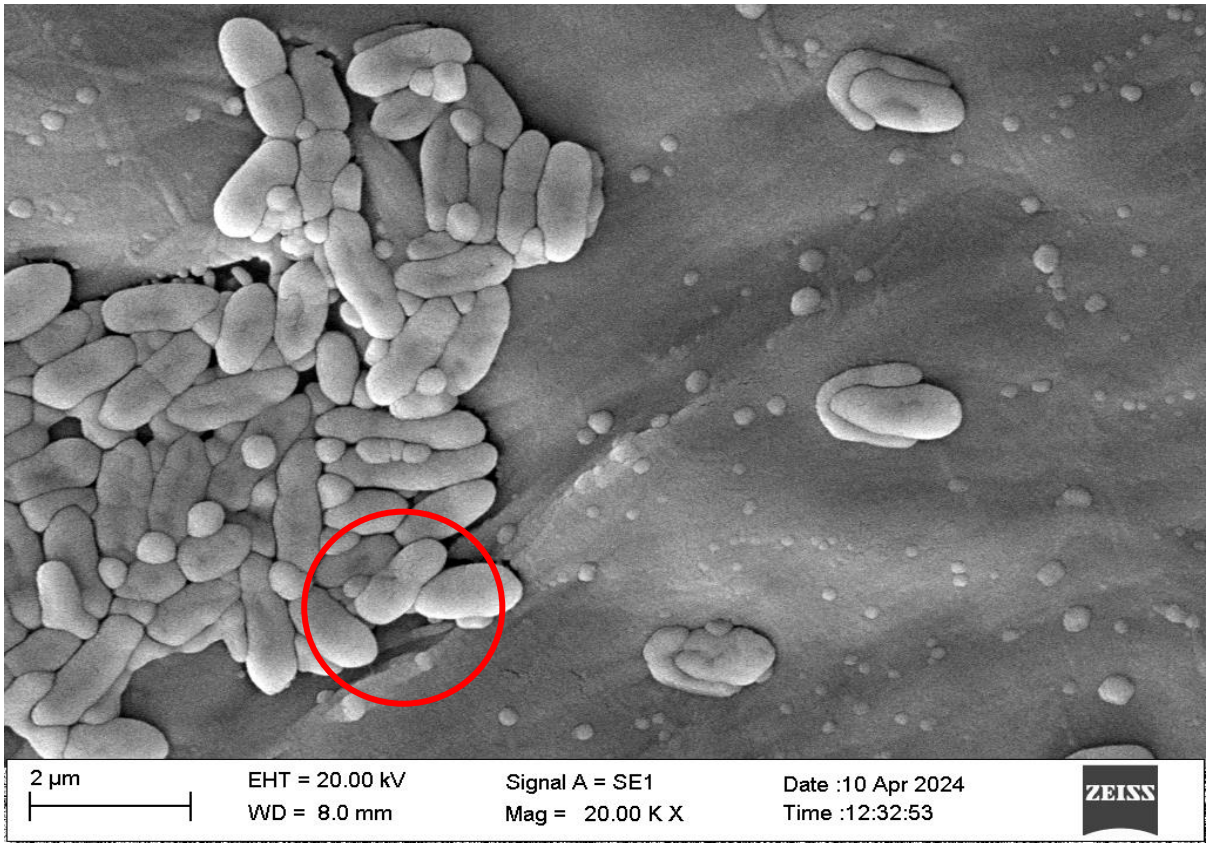

Figure 4.13: SEM photograph of RS1M2 culture showing adherence to plastic.

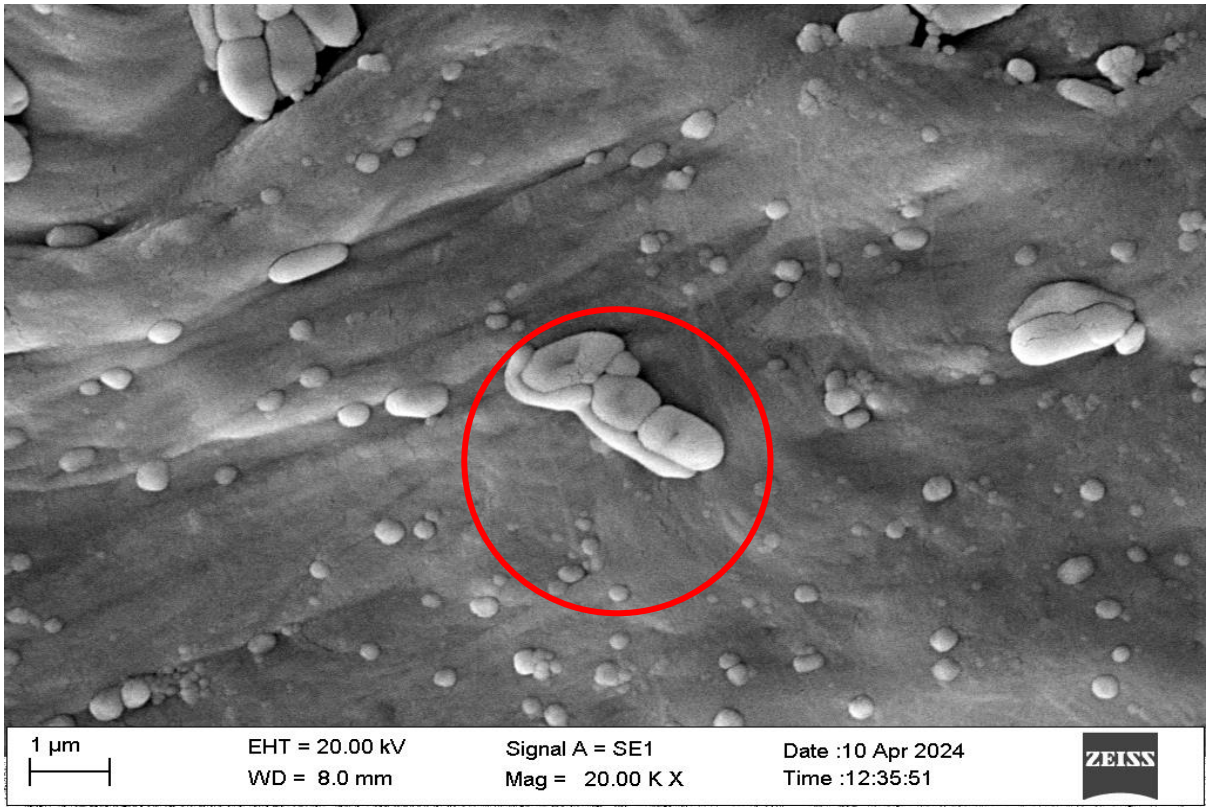

Figure 4.14: SEM photograph of RS1M2 culture showing adherence to plastic.

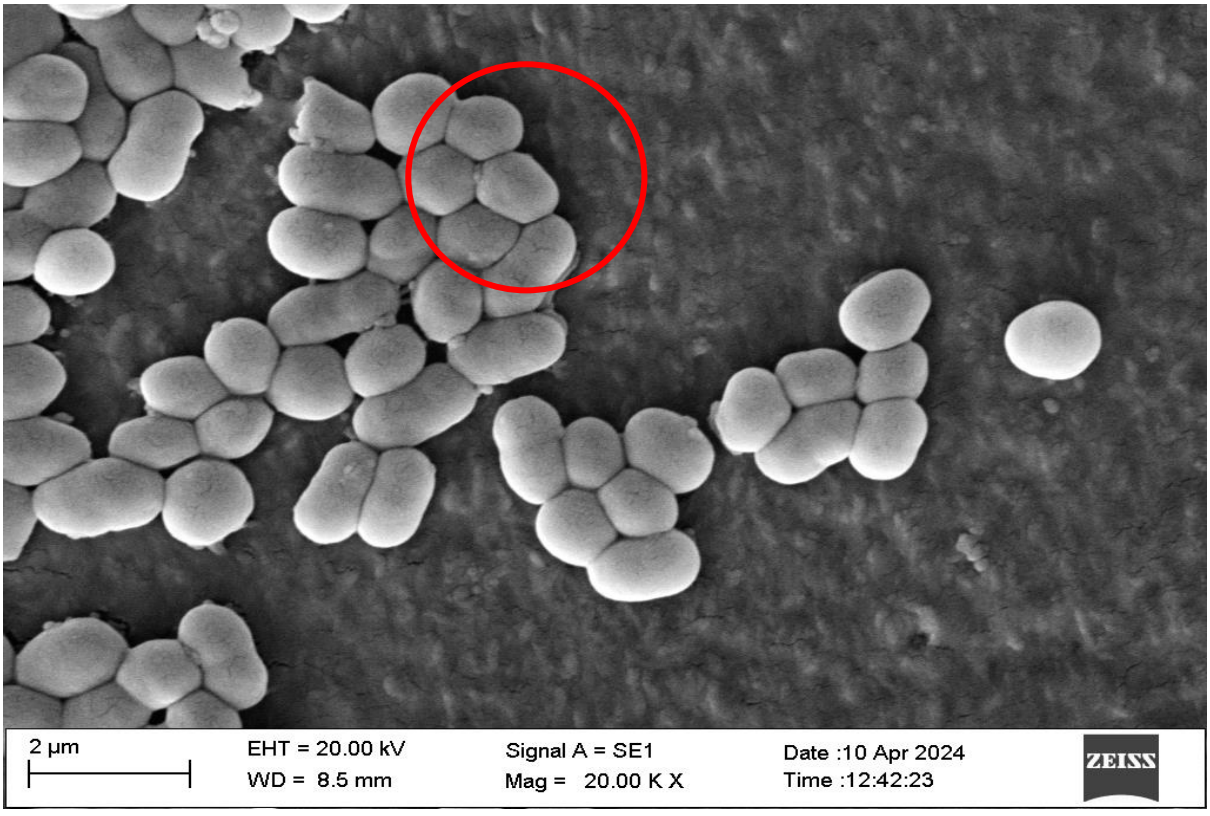

Figure 4.15: SEM photograph of RS2T2 culture showing adherence to plastic

4.2 DISCUSSION

Pathogens were tentatively identified as *Vibrio cholerae*-Like Organisms (VCLO) showing yellow colony morphology around the plastic sample and *Vibrio parahaemolyticus*-Like Organisms (VPLO) showing bluish-green colony on the same media plates on some of the samples collected from Ribandar and Chorao mangroves. *V. cholerae* is an important pathogenic species, because of the production of a potent enterotoxin, the cholera toxin (CT), that disrupts the ion transport of intestinal epithelial cells (Pruzzo et al., 1988) The subsequent loss of water and electrolytes leads to severe diarrhea and vomiting, distinctive characteristics of cholera, and results in severe dehydration (Pruzzo et al. 1988). However, the presence of the cholera toxin gene in the VCLO isolates reported in this study needs to be verified.

Bacteria belonging to family Enterobacteriaceae on media like XLD and EMB were also observed. In an earlier study by Poharkar et al. (2016), a total of 150 samples of water, sediment, and biota were analyzed from ten mangrove ecosystems in Goa, India. Total viable counts of pathogens such *as E. coli, Listeria, Salmonella* and *Vibrio* spp. ranged from 1.25 to 3.9 X 10^3 cfu /mL, and had been identified based on the typical colony morphology on selective media as follows: purple-colored colonies with a green metallic sheen were noted as *E. coli* on EMB agar; green colonies with a black center as *Salmonella* spp. on Hektoen Enteric agar; grey greenish colonies as *Listeria* spp. on PALCAM; and yellow and green colonies as *Vibrio* spp. on TCBS. *Salmonella* counts were the highest at 3.1 to 3.9 X 10^3cfu/mL. In fact, Poharkar et al. (2016) emphasized the role of mangrove habitats as an adopted habitat for pathogenic *Salmonella* species. However, in this study, *Salmonella* was not reported in the plastic debris samples from both Ribandar and Chorao mangroves.

Isolates belonging to the family Enterobacteriaceae were observed on the selective media plates inoculated with the plastic samples from Ribandar and Chorao mangroves i.e. yellow colony presence on MSA media marked the presence of *Proteus mirabilis*, yellow

colony on XLD indicated the presence of *Escherichia coli*. A study by Sangodkar et al. (2020) the prevalence of indicator and pathogenic bacterial groups in water and sediments in OSZ-offshore, ISZ-inshore, IEZ-inner estuary, and UEZ-upper estuary along the river Chapora, central west coast of India, which is influenced by anthropogenic inputs, analyzed the prevalence of indicator and potential pathogenic bacterial groups in water and sediment samples. Counts of indicator bacterial groups such as total coliforms and *Escherichia coli*–Like organisms in water sample ranged from nondetectable (ND) to 10^3 colony-forming units (CFU)/ mL. In contrast, their abundance in the sediments was six orders magnitude higher than that in water (ND to 10^9 CFU/g). The abundance of potential pathogenic bacteria in water and sediment samples ranged from ND to 10^3 CFU/mL CFU/g respectively, with *Shigella*-Like Organisms (SHLO) being the most abundant. In the surface waters, SHLO and *Pseudomonas aeruginosa*–Like Organisms (PALO) and in bottom waters, *Vibrio parahaemolyticus*–Like Organisms and PALO increased progressively from OSZ to UEZ. In contrast, *Proteus/Klebsiella*-Like Organisms (PKLO) showed a reverse trend.

In my study, a total number of 73 bacterial isolates from both the sampling sites were screened for Crystal Violet Assay using 24 well microtiter plates composed of polystyrene. 67 cultures showed OD values higher than 0.5 and 44 cultures showed O.D values greater than 1, indicating that many of the bacterial pathogens from mangrove plastic debris of Chorao and Ribandar mangroves are capable of forming biofilm and exhibiting strong adherence to polystyrene. In a study by Abdallah et al. (2009), 9 *Vibrio* strains were screened for their adherence to polystyrene microplate plates using crystal violet assay. The results showed that only *V. alginolyticus* ATTC 17749 was able to form biofilm (OD570=0.532) and was considered low-grade positive, whereas all the other tested strains did not show any biofilm formation. They also discussed that *Vibrio* is a food-borne pathogen and it can produce biofilm on abiotic surfaces as well as cells. *Vibrio* produce exopolymers that are responsible for the

resistance of these species to desiccation, predation and toxic chemicals. Exopolymers are also considered to be involved in the first steps of biofilm formation (Muller et al., 1993).

The fifteen bacterial isolates showing highest O.D values in the Crystal violet assay were analyzed further, for antibiotic resistance profiles, Gram character and siderophore production. In my study, only one culture showed resistance to Ampicillin i.e. culture RS5T1 which was tentatively identified as *Vibrio cholerae* Like Organism (VCLO) on a plastic sample from Ribandar Mangroves. None of the cultures were resistant to Streptomycin, which can state that Streptomycin is one of the best antibiotics out of the four (Ampicillin, Chloramphenicol, Nalidixic acid, and Streptomycin). This can be due to the different modes of action of Antibiotics. Ampicillin is a beta-lactam antibiotic that inhibits bacterial cell wall synthesis by binding to penicillin-binding proteins (PBPs), leading to cell lysis and death. Chloramphenicol inhibits protein synthesis by binding to the 50S ribosomal subunit, preventing peptide bond formation. Nalidixic acid is a quinolone antibiotic that interferes with bacterial DNA synthesis by inhibiting DNA gyrase, leading to DNA damage and cell death. Streptomycin is an aminoglycoside antibiotic that disrupts protein synthesis by binding to the 30S ribosomal subunit, leading to misreading of mRNA (Willey et al., 2014). The susceptibility of the bacterial isolates to streptomycin in this study is intriguing and differs from the results reported by Jalal et al., (2010) in their study conducted at Tanjung Lumpur mangrove swamp in Malaysia. Bacteria were isolated from mangrove soil samples and their resistance was tested against antibiotics. Identified bacteria were *Aeromonas hydrophila* group 1 and 2, *Escherichia coli* 1, *Chryseomonas luteola*, *Chromobacterium violaceum*, *Pseudomonas aeruginosa*, *Serratia rubudaea*, *Klebsiella pnuemoniae* and *Enterobacter cloacae*. The identified bacteria were introduced to fourteen different antibiotics to determine their bacterial susceptibility. All the isolates showed 100% resistance towards β -lactam antibiotics (ampicillin, amoxicillin and penicillin), vancomycin, sulphafurazole, gentamicin, erythromycin, tetracycline, novobiocin, clindamycin and bacitracin, indicating the presence of bacterial amidases and β-lactamases in

the bacteria which inhibit the action of β - lactam antibiotics. Bacteria isolated from mangrove soil showed 66.7 and 77.8% resistance against chloramphenicol and streptomycin, respectively (Jalal et al., 2010).

In my study ten out of fifteen cultures were found to be positive and the rest of the five were negative for siderophore production from which tentatively identified *Vibrio cholerae-like* organism (VCLO) and one out of two *Vibrio parahaemolyticus* like organisms (VPLO) were positive for siderophore production.Siderhores are low-weight, high-affinity iron chelating molecules produced in response to iron deficiency by Gram-positive and Gram-negative bacteria which are also known as essential virulence factor of bacteria. (Khasheii et al.,2021).

A study by (Pallvi et al., 2023) Bacterial samples were isolated from the saline soil of the Sundarban mangroves. A total of 156 bacterial samples were isolated, six out of 20 isolates were able to create halo zones in the CAS-blue agar assay, indicating that they were able to produce siderophore. Siderophores produced by these halotolerant bacteria in iron deficient environment help in the process of iron sequestration and solubilization, and is an advantage to pathogenic microorganisms, as in this study.

The initial stage of pit formation around the bacterial isolate was observed in SEM around some of the bacterial cultures in my study. One of the cultures on plastic formed an unidentifiable layer around the culture. SEM analysis also identified the adherence of bacterial isolates to the plastic surfaces, bacterial cultures were seen colonizing the surfaces even after two days of incubation period. A study by (Zettler et al., 2013), SEM photomicrographs from North Atlantic revealed a rich bacterial microbiota inhabiting both Polypropylene (PP) and Polyethylene (PE) samples. Cell counts from random images identified over 50 distinct morphotypes covering between 0 and 8% of the plastic surface area. Particularly intriguing were round cells approximately 2 μm in diameter found embedded in pits on the PMD surface

were observed by (Zettler et al., 2013). SEM revealed that samples were prevalently colonized by prokaryotic-sized assemblages in both PE and P. In some cases, eukaryotic microorganisms, such as pennate diatoms (often fragmented) were observed. Rod-shaped cells were more prevalent than coccoid-shaped microorganisms and often occurred in patches, and including dividing cells, a finding that suggested an active microbial growth. Microbial “hot-spots” on plastics mainly occurred when cracks and pits were observed on the plastic surface (Basili et al., 2020).

4.3 CONCLUSION

The findings from this study underscore the significant threat posed by plastic pollution in mangrove ecosystems, particularly the presence of harmful bacterial pathogens associated with plastic debris. The tentative identification of pathogenic bacteria, such as *Vibrio* spp. and bacteria belonging to Enterobacteriaceae on plastics within mangrove sediments and their strong adherence to plastic (as evidenced by high OD values in the crystal violet assay), highlights the ability of plastic debris to facilitate the introduction and spread of potentially harmful microorganisms into these sensitive environments As these plastics float and are carried by the water to different places can disrupt the delicate balance of the microbial community, leading to the proliferation of certain species and the suppression of others. This can have cascading effects on the overall health and functioning of the mangrove ecosystem. Addressing the issue of plastic pollution and its associated microbial threats in mangrove environments will require a multifaceted approach, including enhanced waste management strategies, improved monitoring and surveillance, and the development of innovative solutions to prevent and remediate plastic contamination. By prioritizing the protection and restoration of these vital coastal ecosystems, we can safeguard the delicate balance of life that they support and ensure a sustainable future for both the environment and the communities that depend on it.

REFERENCES

- Alongi, D. M. (2008). Mangrove forests: Resilience, protection from tsunamis, and responses to global climate change. *Estuarine, Coastal and Shelf Science*, *76*(1), 1–13. https://doi.org/10.1016/j.ecss.2007.08.024.
- Arora, N. K., & Verma, M. (2017). Modified microplate method for rapid and efficient estimation of siderophore produced by bacteria. *3 Biotech*, *7*(6), 381. https://doi.org/10.1007/s13205-017-1008-y.
- Barboza, L. G. A., & Gimenez, B. C. G. (2015). Microplastics in the marine environment: Current trends and future perspectives. *Marine Pollution Bulletin*, *97*(1–2), 5–12. https://doi.org/10.1016/j.marpolbul.2015.06.008.
- Carrasco-Acosta, M., Santos-Garcia, M., & Garcia-Jimenez, P. (2022). Marine bacteria associated with colonization and alteration of plastic polymers. *Applied Sciences*, *12*(21), 11093. https://doi.org/10.3390/app122111093
- Carson, H. S., Nerheim, M. S., Carroll, K. A., & Eriksen, M. (2013). The plastic-associated microorganisms of the North Pacific Gyre. *Marine Pollution Bulletin*, *75*(1–2), 126–132. https://doi.org/10.1016/j.marpolbul.2013.07.054.
- Carvalho Ferreira, H., & Lôbo-Hajdu, G. (2023). Microplastics in coastal and oceanic surface waters and their role as carriers of pollutants of emerging concern in marine organisms. *Marine Environmental Research*, *188*, 106021. https://doi.org/10.1016/j.marenvres.2023.106021.
- De Tender, C. A., Devriese, L. I., Haegeman, A., Maes, S., Ruttink, T., & Dawyndt, P. (2015). Bacterial community profiling of plastic litter in the belgian part of the North Sea. *Environmental Science & Technology*, *49*(16), 9629–9638. https://doi.org/10.1021/acs.est.5b01093.

- Dey, S., Rout, A. K., Behera, B. K., & Ghosh, K. (2022). Plastisphere community assemblage of aquatic environment: Plastic-microbe interaction, role in degradation and characterization technologies. *Environmental Microbiome, 17(1),* 32. https://doi.org/10.1186/s40793-022-00430-4.

- Donato, D. C., Kauffman, J. B., Murdiyarso, D., Kurnianto, S., Stidham, M., & Kanninen, M. (2011). Mangroves among the most carbon-rich forests in the tropics. *Nature Geoscience*, *4*(5), 293–297. https://doi.org/10.1038/ngeo1123.

- Du, Y., Liu, X., Dong, X., & Yin, Z. (2022). A review on marine plastisphere: Biodiversity, formation, and role in degradation. *Computational and Structural Biotechnology Journal*, *20*, 975–988. https://doi.org/10.1016/j.csbj.2022.02.008.

- Gall, S. C., & Thompson, R. C. (2015). The impact of debris on marine life. *Marine Pollution Bulletin, 92*(1–2), 170–179. https://doi.org/10.1016/j.marpolbul.2014.12.041.

- Galloway, T. S., Cole, M., & Lewis, C. (2017). Interactions of microplastic debris throughout the marine ecosystem. *Nature Ecology & Evolution*, *1*(5), 1–8. https://doi.org/10.1038/s41559-017-0116.

- Geyer, R., Jambeck, J. R., & Law, K. L. (2017). Production, use, and fate of all plastics ever made. *Science Advances*, *3*(7), e1700782. https://doi.org/10.1126/sciadv.1700782.

- Jacquin, J., Cheng, J., Odobel, C., Pandin, C., Conan, P., Pujo-Pay, M., Barbe, V., Meistertzheim, A.-L., & Ghiglione, J.-F. (2019). Microbial ecotoxicology of marine plastic debris: A review on colonization and biodegradation by the "plastisphere." *Frontiers in Microbiology*, *10*, 865. https://doi.org/10.3389/fmicb.2019.00865.

- Keswani, A., Oliver, D. M., Gutierrez, T., & Quilliam, R. S. (2016). Microbial hitchhikers on marine plastic debris: Human exposure risks at bathing waters and beach environments. *Marine Environmental Research*, *118*, 10–19. https://doi.org/10.1016/j.marenvres.2016.04.006.

- Kirstein, I. V., Kirmizi, S., Wichels, A., Garin-Fernandez, A., Erler, R., Löder, M., & Gerdts, G. (2016). Dangerous hitchhikers? Evidence for potentially pathogenic *Vibrio* spp. on microplastic particles. *Marine Environmental Research*, *120*, 1–8. https://doi.org/10.1016/j.marenvres.2016.07.004.

- Kour, H., Khan, S. S., Kour, D., Rasool, S., Sharma, Y. P., Rai, P. K., Singh, S., Chaubey, K. K., Rai, A. K., & Yadav, A. N. (2022). Microbes mediated plastic degradation: A sustainable approach for environmental sustainability. *Journal of Applied Biology & Biotechnology*. https://doi.org/10.7324/JABB.2023.110515.

- Kwon, J., Chang, S., Hong, S. H., & Shim, W. J. (2017). Microplastics as a vector of hydrophobic contaminants: Importance of hydrophobic additives. *Integrated Environmental Assessment and Management*, *13*(3), 494–499. https://doi.org/10.1002/ieam.1906.

- Mossop, J. (2018). The relationship between the continental shelf regime and a new international instrument for protecting marine biodiversity in areas beyond national jurisdiction. *ICES Journal of Marine Science*, *75*(1), 444–450. https://doi.org/10.1093/icesjms/fsx111.

- Naik, M. M., Bhangui, P., & Bhat, C. (2017). The first report on Listeria monocytogenes producing siderophores and responds positively to N-acyl homoserine lactone (Ahl) molecules by enhanced biofilm formation. Archives of Microbiology, 199(10), 1409–1415. https://doi.org/10.1007/s00203-017-1416-8.

- Oberbeckmann, S., Kreikemeyer, B., & Labrenz, M. (2018). Environmental factors support the formation of specific bacterial assemblages on microplastics. *Frontiers in Microbiology*, *8*, 2709. https://doi.org/10.3389/fmicb.2017.02709.

- Poharkar, K. V., Kerkar, S., D'Costa, D., Doijad, S., & Barbuddhe, S. B. (2016). Mangrove ecosystems: An adopted habitat for pathogenic *Salmonella* spp. *Water Environment Research*, *88*(3), 264–271. https://www.jstor.org/stable/44134405.

- Rosenberg, A., Weinberger, M., Paz, S., Valinsky, L., Agmon, V., & Peretz, C. (2018). Ambient temperature and age-related notified Campylobacter infection in Israel: A 12-year time series study. *Environmental Research*, *164*, 539–545. https://doi.org/10.1016/j.envres.2018.03.017.

- Silva, M. M., Maldonado, G. C., Castro, R. O., De Sá Felizardo, J., Cardoso, R. P., Anjos, R. M. D., & Araújo, F. V. D. (2019). Dispersal of potentially pathogenic bacteria by plastic debris in Guanabara Bay, RJ, Brazil. *Marine Pollution Bulletin*, *141*, 561–568. https://doi.org/10.1016/j.marpolbul.2019.02.064.

- Sun, R., Liu, Y., Li, T., Qian, Z.-J., Zhou, C., Hong, P., Sun, S., & Li, C. (2023). Plastic wastes and surface antibiotic resistance genes pollution in mangrove environments. *Environmental Monitoring and Assessment*, *195*(6), 634. https://doi.org/10.1007/s10661-023-11312-8.

- Thompson, R. C., Moore, C. J., Vom Saal, F. S., & Swan, S. H. (2009). Plastics, the environment and human health: Current consensus and future trends. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *364*(1526), 2153–2166. https://doi.org/10.1098/rstb.2009.0053.

- Urbanek, A. K., Rymowicz, W., & Mirończuk, A. M. (2018). Degradation of plastics and plastic-degrading bacteria in cold marine habitats. *Applied Microbiology and Biotechnology*, *102*(18), 7669–7678. https://doi.org/10.1007/s00253-018-9195-y.

- Wright, R. J., Erni-Cassola, G., Zadjelovic, V., Latva, M., & Christie-Oleza, J. A. (2020). Marine Plastic Debris: A New Surface for Microbial Colonization. *Environmental science & technology*, *54*(19), 11657–11672. https://doi.org/10.1021/acs.est.0c02305.

- Yu, M.-H., Ding, G.-D., Gao, G.-L., Zhao, Y.-Y., & Sai, K. (2019). Hysteresis resulting in forestry heat storage underestimation: A case study of plantation forestry in northern China. *Science of The Total Environment*, *671*, 608–616. https://doi.org/10.1016/j.scitotenv.2019.03.340.

- Zadjelovic, V., Wright, R. J., Borsetto, C., Quartey, J., Cairns, T. N., Langille, M. G. I., Wellington, E. M. H., & Christie-Oleza, J. A. (2023). Microbial hitchhikers harbouring antimicrobial-resistance genes in the riverine plastisphere. *Microbiome, 11*(1), 225. https://doi.org/10.1186/s40168-023-01662-3.

- Zeenat, Elahi, A., Bukhari, D. A., Shamim, S., & Rehman, A. (2021). Plastics degradation by microbes: A sustainable approach. *Journal of King Saud University - Science, 33*(6), 101538. https://doi.org/10.1016/j.jksus.2021.101538.

- Zettler, E. R., Mincer, T. J., & Amaral-Zettler, L. A. (2013). Life in the "plastisphere": Microbial communities on plastic marine debris. *Environmental Science & Technology, 47*(13), 7137–7146. https://doi.org/10.1021/es401288x.

APPENDICES

Appendix I: Media composition

ZMA (Zobel Marine Agar)

Ingredients	**Grams/litre**
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

EMB(Eosin Methylene Blue) 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

TCBS (Thiosulfate Citrate Bile Sats Sucrose) 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

XLD (Xylose Lysine Deoxycholate) 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

MSA (Mannitol Salt Agar)

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

ZMB (Zobel Marine Broth)

Ingredients	**Grams/litre**
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

Luria Bertani Agar

Ingredients	**Grams/litre**
Tryptone	10.000
Yeast extract	5.000
Sodium chloride	10.000
Agar	15.000
Final pH	7.5 ±0.2

Chrome azurol S

Ingredients	**Grams/litre**
CTAB (Stock)	6ml
Distilled water	40ml
Ferric Chloride (Stock)	1.5ml
Hydrochloric acid	6.5ml
Anhydrous piperazine	4.3h

Appendix II: Reagents preparation

Preparation of 1L Phosphate Buffered Saline (PBS) (1x)

Components	**gm/L**
Sodium chloride (NaCl)	8
Potassium chloride (KCl)	0.2
Disodium hydrogen phosphate (Na_2HPO_4)	1.44
Potassium dihydrogen phosphate (KH_2PO_4)	0.24

Adjust pH to 7.4

Preparation of 100 mL 50% ethanol in Phosphate Buffered Saline (PBS)

Add 50 mL of ethanol in 50 mL PBS

Preparation of ethanol series

50%

Add 50 mL ethanol and 50 mL distilled water.

75%

Add 75 mL ethanol and 25 mL distilled water.

85%

Add 85 mL ethanol and 15 mL distilled water.

95%

Add 95 mL ethanol and 5 mL distilled water.

1% Crystal Violet

Ingredient	Grams/Litre
Crystal Violet	0.1
Distilled Water	100ml

30% Acetic acid

Add 30ml acetic acid in 70ml distilled water.