Investigation of Microplastic Associated Bacterias from Cacra Beach, Goa, India

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By

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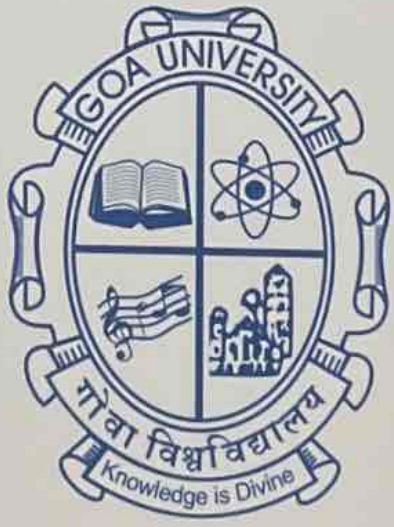

GOA UNIVERSITY
DATE: APRIL 2023

COMPLETION CERTIFICATE

This is to certify that the dissertation / internship report " **INVESTIGATION OF MICROPLASTIC ASSOCIATED BACTERIA FROM CACRA BEACH, GOA INDIA.**" is a bonafide work carried out by **Ms. Shaaratha V Rao.** under my supervision/mentorship in partial fulfilment of the requirements for the award of the degree of **Master's Degree** in the Discipline **Microbiology programme** at the **SCHOOL OF BIOLOGICAL SCIENCES AND BIOTECHNOLOGY**, Goa University.

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DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation / Internship report entitled, " Investigation of Microplastic Associated Bacteria from Cacra beach, Goa , India" is based on the results of investigations carried out by me in the Microbiology programme at the School of Biological Sciences and Biotechnology, Goa University under the Supervision of Dr. Milind Mohan Naik and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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Shaaratha V Rao.

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(Lisa Stentvedt, conservation talk)

INTRODUCTION

Introduction:

The scientific community paid little attention to the first discovery of plastic litter in the ocean in the early 1970s. In the following years, research interest in animal entanglement and its impact on it, such as digestive and reproductive impairment , has continued to increase. A high-priority research area in marine biology has been assigned to this topic following recent reports of unexpected occurrences of plastic debris in the North Pacific Rotation. The presence of small plastic debris is of particular concern.

1.1 Plastics:

The current annual global demand for plastics is estimated at US$245 million, increasing steadily in recent years. Plastics are ideal for a multitude of applications because they are adaptable, lightweight, durable, and optionally transparent. These materials offer excellent packaging due to their affordability, superior moisture and oxygen resistance qualities, bio-inertia and light weight. Plastic packaging that is more affordable and of equal or superior design is replacing traditional materials such as glass, metal and paper. As a result, consumer packaging materials such as disposables

commonly found in beach debris are made up of about a third of all plastic produced. (Andrady, 2011)

Polyethylene (PE), polypropylene (PP), polystyrene (PS), poly (ethylene terephthalate) (PET) and poly(vinyl chloride) are some of the major polymers used in packaging (PVC). Future ocean plastic waste will increase due to fishing, recreational and marine water use, demographic changes favouring migration to coastal areas, and other factors. About 80% of plastic waste comes from land-based sources, including beach litter. Now that all fishing gear is plastic, they are frequently lost or carelessly discarded at sea while being used by fishing fleets around the world. The majority of fishing gear applications use nylon and polyolefins (PE and PP). The fishing industry is responsible for about 18% of marine plastic litter found in the ocean environment. A major source of plastic waste in the ocean can also come from aquaculture operations. Beach litter and other land-based sources make up most of the remaining material. Unintended losses in transit or runoff from processing plants are common routes by which virgin plastic pellets, a common component of debris, enter the sea.

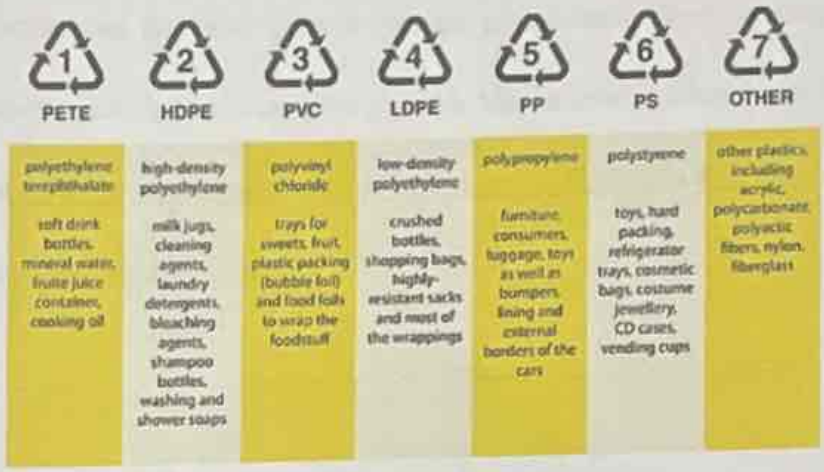

Fig.1.1 types of plastics (Earth Easy)

1.2 Garbage patch:

Due to the effects of ocean currents and increase in plastic pollution a gyre of marine debris particles forms a garbage patch. Oceanic gyres are where the currents are weakest and once these man made plastic and other waste debris reach the ocean they start to float and end up in the middle of these gyres. "For every square mile of ocean there are about 46000 pieces of plastic"- estimated by the United Nations environmental programme. The best known and the largest garbage is the Great Pacific Garbage patch which mostly contains large density debris and plastics. Other patches that have been

identified are the North Atlantic garbage patch, south Atlantic garbage patch, south pacific garbage patch, Indian ocean garbage patch. Most widely monitored gyre is in the pacific ocean for insights of accumulation of plastics. The source of plastic is necessary to be known for effective cleanup.(Lebraton, 2022)

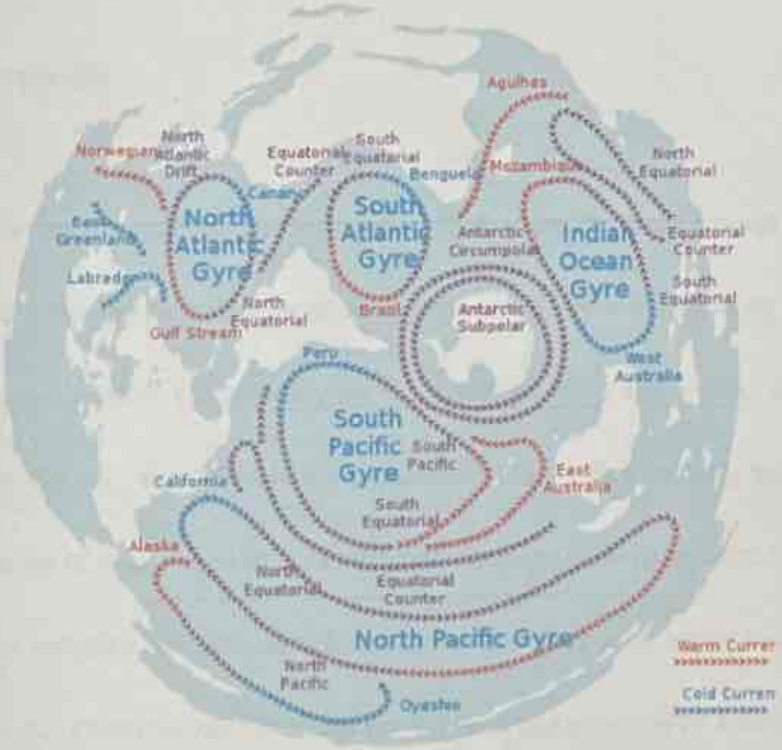

Fig. 1.2 garbage patches around the world. (Avsa, 2009)

1.3 Indian Ocean Garbage Patch:

One of the five ocean gyres is the Indian ocean gyre. This patch in particular is filled with plastic debris. This field in particular is not a continuous debris field. In this

The primary particles are not visible to the naked eye and the concentration is estimated to be around 10000 particles per square kilometre. Since it has around 90% plastic debris it has a detrimental effect on the marine ecosystem. Since it has a transient accumulation of plastic debris it poses a serious threat to marine biodiversity in the coastal region. (Riskas, 2019)

1.4 Microplastics:

The term "microplastics" was coined to describe the large amount of small plastic(100 nm-5mm) waste that is present in all seas of the world. By breaking larger plastic objects, ropes and synthetic textiles, mechanically by the action of wind and waves, or by photo-oxidation caused by the sun, microplastics are created; these are called primary microplastics. They are also made up of micro-manufactured materials such as micro-beads used in cosmetics and body washes to exfoliate the skin, which run down the drain after application, or micro-particles created by 3D printers or used in paints and coatings that go to sea by a similar route and are also called secondary microplastics. With the addition of colourants, plasticizers, hardeners, softeners, UV screeners and antibacterial agents, plastics can be transformed into a multitude of colours, shapes and materials as a result, we are able to produce many of the items we use every day in modern society, such as plastic bags and bottles, packaging materials, computer screens, plant pots, building materials, clothing, disposable medical devices, and even

medicines themselves. As a result, when we use the phrase "microplastics," we mean a complicated mixture of forms and sizes, fragments, fibres, and particles generated from a wide variety of polymer types and chemical additives.

Fig. 1.3 small fragments of plastics found on the beach. (Return to Now, 2017)

1.5 Effects of plastic on marine life:

Plastic that are discarded can end up suspended in the marine environment for a long time. The plastic that is suspended can be consumed by turtles since their diet included jellyfishes and the plastic covers resemble the jelly fishes. Many species, one

among them Black footed Albatross, have been found to feed their young ones with plastic granules. Major threat to marine life is entanglement which may eventually lead to the death of the animal without any intervention.

Most natural and nanoparticles in sea water are <100 nm in size. The majority of the filter feeders from small nano- Zooplanktons to the enormous Baleen Whales interact with these sizes of particles without any adverse effect. But since the microplastic also ranges within the same range of size the interaction between them is a problem. These range of particles are a staple for organisms like Pacific Krill. For experimental purposes microbeads are used in the feeding research. These were then found that they ingest both the zooplankton and microplastic beads. Since these organism do not have any mechanism to breakdown or adsorb the particle they become bio-inert and now pose a major threat since some of the organisms are used as delicacy for human consumption. This plastic waste then finally enters the human body and cause major health issues.

1.5 Formation of the microplastics in marine environment:

1. Plastic entering the ocean.
2. Deposition on the shore.
3. The plastics then are fragmented and broken down through physico chemicals into microplastics and nano plastics.

4. Through Mechanical stress,
5. Through photodegradation,
6. Possibly through biodegradation.

1.6 Various pathways for degradation of plastic in marine environment-

1. **Fragmentation**:

The breakdown of parent plastic into daughter particles is led by the structural weakness and mechanical integrity loss. The fragmentation process leads to the changes in the size of the plastic. it does not eliminate the plastic from the environment. It may help as a positive catalyst for the physicochemical and biochemical reactions as the surface to volume ratio increases with smaller particles. The plastic particles >2mm that are exposed to sunlight wither faster and fragment. They form cubic particles which are then easier to be broken down even further - Nano plastic particles.(Wayman & Niemann, 2021)

2. **photooxidation**:

The physical properties of the plastics are changed through this process at the molecular level. The plastic is made brittle due to the loss of mechanical properties of the polymer. the photooxidation has some main reactions such as,

1. initiation
2. propagation
3. termination.

The above reactions lead to the changes in the molecular weight and its distribution in the polymer. This results in the loss of mechanical properties. photooxidation along with fragmentation can lead to the formation of small molecules and even nanoparticles. This process is considered an important sink for plastics.

4. **Biodegradation**:

This process removes the plastic particle from the environment. The carbon from plastic is assimilated and mineralized by microorganisms. factors such as temperature and nutrient availability might influence the biodegradation in oceans. The daughter plastics that are derived through the photooxidation and fragmentation can be biodegradation by

microorganisms. The plastic degradation can be carried out by microorganisms only, i.e., the initial degradation into smaller particles and the further oxidation of it can be done by microbes. The microbes carry out the biodegradation through 2 steps. One is indirect in which the products from the microbes can affect the plastic particles. The other method is indirect methods in which the plastic serves for the growth of the microorganism. The plastic can be a carbon source and energy source for some bacteria and some fungi. (Wayman & Niemann, 2021)

1.7 The interaction between microplastics and microorganisms:

In the biogeochemical cycle the microorganisms play an integral role and are associated with microplastics. The introduction of plastic litter has created a new ecological niche in the marine environment which is termed as plastisphere. Some organisms such as dinoflagellates, diatoms, isopods, coco lithotrophs, bryozoans, barnacles, cyanobacteria, heterotrophic bacteria, and fungi have been found on plastics. Hypoplastic microorganisms are diverse and distinct in their community composition. The organisms differ even phenotypically.(Rogers et al., 2020)

1.8 biofilm formation and biofouling:

The fate and potential effect of microplastics(<5mm) in the marine environment are modified by the key process of biofilm formation. The process of weathering also helps in the biofilm formation because it increases the surface area for colonisation which can also shield the plastic debris from ultraviolet light. The selective attachment of microorganisms occurs once after the plastic is released into the marine environment which then facilitates, and interspecific competition occurs within the community. (Rummel et al., 2017)

1.9 Antibiotic resistance:

The increase in the Multi Drug Resistant bacteria is a great threat to human life due to its decreased efficiency of the antibiotics. In the environment human activities such as industrial waste, mining waste, agriculture, aquaculture, and sewage pollution are considered to be a hotspot for the development of antibiotic resistance in bacteria. Antibiotics that are used for livestock, residues of antibiotics in human faeces and also from pharma industries contaminate the environment.(Young, 1993)

1.10 Bacteria acquire antibiotic resistance in several ways:

Due to antibiotic stress the bacteria undergo several different genotypic and phenotypic chances as a coping mechanism. The bacteria that produce the antibiotic naturally becomes resistant to it due to the genes that confer the resistance. The other bacteria acquire the resistance to the exposure to the antibiotics. Due to horizontal transfer the resistance can be acquired from one bacteria to the other through process of i) transduction ii) transformation, iii) conjugation or the mutation of antibiotic targets can also be a cause for resistance by activation of efflux pump (5 types of efflux transporters) (Imran et al., 2019)

1.11 Metal resistance:

Metal pollution in the environment is contributed by the inclusion of metals for various purposes such as feed in aquaculture, fertilisers , paints, construction industry etc, for living organisms metal is essential for various physiological functions of the cell at a particular concentration. Metals such as lead and mercury, cadmium are toxic heavy metals that are Biologically non-essential and it contaminates various terrestrial and coastal regions. It is very persistent in the environment and hence they bioaccumulate and

enter the food chain and are a great threat to the terrestrial and marine ecosystems. (Imran et al., 2019)

1.12 Metal resistance mechanisms in bacteria:

For their sustainability the bacteria have acquired metal resistance mechanisms to avoid the adverse effects of the metal. By various methods such as, i) intracellular sequestration, ii) extracellular sequestration, iii) bioprecipitation and biotransformation, iv) morphology alteration and pigment production, v) efflux mechanisms, vi) biofilm formation. (Imran et al., 2019)

1.13 co-selection of antibiotic resistance and metal resistance:

The antibiotic resistance by the microorganism can be achieved in several ways such as reduced membrane permeability for the antibiotics, mutation of the gene encoding the target of the antibiotic, efflux of the antibiotic outside of the cell, antibiotics inactivation, extracellular sequestration and biofilm formation. For the metal resistance also, microorganisms are found to use similar strategies. The co- selection can be

achieved by 2 ways that is co-resistance or cross- resistance. Cross resistance can be defined as a single resistance mechanism that is responsible for resistance from different compounds. Co resistance can be defined as two or more resistance conferring genes that are present in the same mobile genetic elements and can be resistant to different compounds simultaneously. (Imran et al., 2019)

1.14 Co- selection - Microplastics is Hotspot :

Microplastics have harmful effects on aquatic organisms and also on humans through the food chain. In recent years studies have revealed that the metals can be absorbed by the microplastics on their surfaces. For heavy metals like copper and zinc they act as vectors. The biofilm formation is a potential threat due to the pathogens that can be associated with it. It is also revealed that not only metals but also antibiotics can also be absorbed on the surfaces of these microplastics. (Imran et al., 2019).

Previous studies:

The microplastic associated bacteria are studied by various scientists. Soem of the studies that are carried out are how the municipal waste water treatment spreads the bacteria, Study of plastic and biofilm, Colonisation mechanisms, Antibiotic resistance, Heavy metal resistance etc,.

The previous studies on microorganisms were done such as *Providencia vermicola* strain SJ2A which was found to sequester lead intracellularly. *Vibrio harveyi* uses phosphatase enzymes to bio precipitate lead. *Bacillus* sp. strain RC607 reduces Hg^{2+} to volatile Hg^{0} by mercury reductase Mer A protein *Pseudomonas aeruginosa* strain 4EA showed changes in cell morphology to deal with lead nitrate present in minimal media. *Pseudomonas putida* onshowed blebbing on its outer membrane when exposed to Cadmium. By Biofilm Formation the organism *Pseudomonas* strain EJO1 could tolerate 7mM cadmium. *Pseudomonas aeruginosa* WI-1 shows multi drug resistance and also lead resistance. It was also found to be tolerating multiple antibiotics and mercury, cadmium. *Pseudomonas stutzeri* strain M-9 shows resistance to mercury and cadmium. *Staphylococcus aureus* shows resistance genes for both penicillin Antibiotic and Mercury which are linked on plasmids. (Imran et al., 2019)

(National Geographic, 2019)

AIMS AND OBJECTIVES

Aims and Objectives:

Plastic is a growing threat to the environment as well as the biodiversity in the marine environment. microplastic is posing a major threat to marine animals as well as humans. since they are harmful to the fishes that consume it And also for the humans that consume the fishes. there is an exponential increase in the antibiotic resistance in microorganisms that are also now steadily gaining resistance to metals.

In the view of this, the following objective were sited:

- Collection of microplastic from Cacra beach.
- Isolation of microplastic associated pathogenic bacteria.
- Identification of microplastic associated bacteria.
- Antibiotic resistance profile of the microplastic associated bacteria.
- Metal (Hg, Cd) resistance in microplastic associated bacteria.
- Plasmid profile of microplastic associated bacteria.

(E&T, 2021)

MATERIALS AND METHODS

materials and methodology:

3.1 collection of microplastics:

The microplastics were collected from the marine environment (cacra beach). the microplastics were identified (<5mm) and collected with sterile forceps and was collected in falcon tubes.

Fig. 3.1 the microplastic collected in the falcon tube, the circle showing the plastic

Media preparation:

The media used are EMB, ZMA, MAC, TCBS, SS, MSA. Media were prepared by weighing the appropriate amount of media from the given amount in readymade media from HIMedia.

3.2 isolation of bacteria associated to microplastics:

For isolating pathogenic bacteria associated with the microplastics 6 medias were used. specific selective and differential medias were used such as MacConkey agar, Eosin methylene blue agar, mannitol salt agar, Thiosulphate citrate bile salt sucrose agar, salmonella shigella agar, Zobell marine agar. The microplastic pieces (<5mm) were carefully placed on the plates in the centre and incubated for 24 hours in an incubator.

Fig. 3.2 The picture shows SS agar, EMB agar, MSA agar, TCBS agar, MAC agar, ZMA agar. Here the microplastic is placed at the centre of the agar plates using sterile forceps.

3.3 identification of the bacteria associated with microplastics:

3.3.1 Biochemical tests for identification- IMViC test:

- **3.3.1.1 Indole test**:

Tryptophan broth was prepared and autoclaved as media for the isolates. 24 hours grown culture was inoculated and was incubated for 24-48 hours. After 24 hours the tubes were taken from the incubator and a few drops of Kovac's reagent (a solution of 4-(dimethylamino) benzaldehyde and hydrochloric acid in n-butanol or amyl alcohol) is commonly used. It is preferred for aerobic cultures. the formation of the red ring after the addition of the reagent marks positive and no change in the tubes is negative. the result was noted.

- **3.3.1.2 Methyl Red test**:

For this test methyl red- VP broth is used. The broth is prepared and autoclaved after which 24 hours grown culture is inoculated in the broth and is incubated for 24 hours. After 24 hours of incubation time the presence of a methyl red indicator will give

red colour for positive results and no colour change for negative results. The results for the isolates are noted down.

❖ **3.3.1.3 Voges- Proskauer test:**

The same as methyl red test MR-VP broth is used. For both the tests it can be prepared together and autoclaved and poured into sterile test tubes. The isolates are inoculated in the medium and incubated for 48 hours. The reagent Barrett's A, and Barrett's B were added and the red colour change indicates positive results and no colour change indicates negative result. the results are noted.

❖ **3.3.1.4 Citrate Utilisation test:**

Simmons citrate agar is used for the test. It has a bromothymol blue indicator which on utilisation of citrate will change its original green colour into blue. The agar is prepared and autoclaved and poured into tubes and is placed at an angle which allows it to form a slant when it solidifies. The isolates are streaked onto the slants and are incubated for 24-48 hours. The change in colour signifies the utilisation and is a positive result.

❖ **Biochemical test kit:**

The biochemical test kit of HIMedia (HiMViC- KB001) was used as per the instructions given in the manual. The culture was grown overnight at 37°C for 18-24 hours. The result for the biochemical and carbohydrate utilisation was recorded and compared with the standard chart.

❖ **3.3.1.5 catalase Test:**

The isolates were smeared in glass slides and air dried. Then 3% hydrogen peroxide solution is taken in a dropper and suspended in the smear. presence of effervescence shows positive results and no effervescence indicates negative results.

3.4 antibiotic susceptibility test:

To check the antibiotic sensitivity of isolated bacteria. Muller Hinton medium is used. The medium is autoclaved and 70 ml of medium is poured into each plate. the cultures were inoculated in Nutrient broth and were incubated for 18 hours at 37°C. This 24-hour culture was taken (0.2ml) and spread plated onto the plate. and HIMedia octa

disc was placed on the plates where already microbial culture was spread plated and this was inverted and incubated for 24 hours at 37 C. The results were noted. Zone of inhibition was noted and measured.

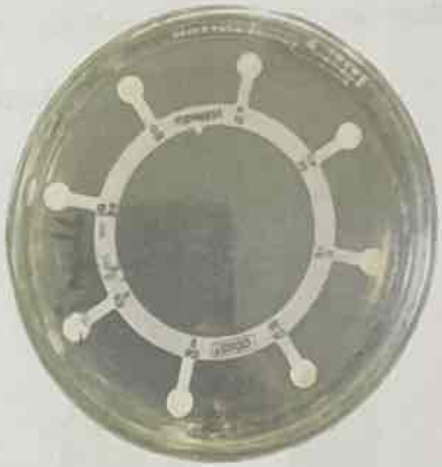

Fig 3.3 antibiotic infused octa disc (make: HI Media)

3.5 metal resistance:

Cadmium sulphate and mercuric chloride were used for checking metal resistance. 5 flasks were taken for each isolate and nutrient broth was prepared and autoclaved. Stock solution of mercuric chloride and cadmium sulphate are prepared using

deionized water.Both media and stock solution are autoclaved separately. From the stock solution different concentrations of metal (Cadmium sulphate and mercuric Chloride separately) 0.1mM, 0.2mM, 0.4mM, 0.5mM, 1mM were calculated and weighed and media was prepared. The exact concentration of the metals required is pipetted out by the C1V1=C2V2 method and added to the media. This was then poured onto plates and the next day the cultures were streaked onto the media and incubated at 37C. The results were observed and interpreted.

Fig. 3.4 stock solutions of HgCl2 and CdSO4 were prepared using deionized water and autoclaved.

3.6 plasmid isolation:

The isolates are inoculated in the broth and are incubated for 24 hours at 37°C.

- In a 2 ml Eppendorf tube 1.5 ml of culture is taken.
- centrifuged at 8000 rpm for 2 minutes.
- The supernatant is discarded and the pellet is retained.
- This process is repeated 2 times to acquire more pellets.
- The pellet is introduced to a resuspension solution of 250 microlitre and vortexed.
- to this 250 microlitre of lysis solution is added. it should not be vortexed but should be mixed by inverting a few times.
- 350 microlitre of neutralising solution is added and mixed immediately by inverting the tubes.
- This is centrifuged for 5 minutes at 12000 rpm.
- supernatant is now pipetted and put through a column filter.
- centrifuged for 1 minute.
- the flow through is discarded.
- 500 microliter of wash solution is added to the column.
- centrifuged for 30-60 seconds.

- the flow through is discarded.
- The process is repeated- 500 microliter of wash solution is added and centrifuged and the flow through is discarded.
- centrifuged for 1 minute.
- The column is now placed in a new Eppendorf tube and a 50 microliter of elution buffer is added and incubated for 2 minutes at room temperature. and then centrifuged for 2 minutes.
- the last step is repeated 2 times
- discard the column and store the plasmid at -20 C

3.6.1 Agarose gel electrophoresis:

- 0.8 g of agarose is taken and suspended in 100 ml of TB buffer (1x concentration)
- This is microwaved for 2 minutes to melt the content.
- After it gets cooled down 25 microliter of Ethidium bromide is added with gloves.
- The tray, comb, and electrophoresis chamber is cleaned and the prepared agarose is poured into the tray and comb is placed.

- After polymerization of the agarose gel the comb is removed and is placed in the chamber. The running buffer is 1x concentration.
- A sample is prepared with placing a drop of dye and the isolated sample is put and 6 microliter is pipetted and put in the wells.
- a ladder of 100 base pairs or 1 kb is also put in a well for standardisation.
- The voltage is set not more than 100 volts and it is switched on.
- It is allowed to run till the dye runs ¾ th of the gel and then it's switched off. The gel is then viewed under the uv- transilluminator.
- The gel is observed for bands and is compared with the ladder.

(CarryWell)

RESULTS AND DISCUSSION

4.1 ISOLATION:

The plastic approx. of 5mm that was placed on the respective selective and differential media and incubated at 37° C for 18-24 hours. The colonies that grew surrounding the plastic were carefully removed using sterile loops and streaked in the respective plates again and incubated in the same conditions and the colonies are observed.

Table 4.1

Isolate	media used	colonies
isolate 1	EMB agar	

isolate 2	SS agar	
isolate 3	ZMA agar	

isolate 4	TCBS agar	
isolate 5	McConkey agar	
no isolate	MSA	no growth in this media.

4.2 Colony characteristic of the isolates:

Table 4.2

Media	EMB	SS	TCBS	Mac	ZMA
TIME	24 hrs	24 hrs	24 hrs	24 hrs	24 hrs
TEMP.	37°C	37°C	37°C	37°C	37°C
SHAPE	circular	circular	circular	circular	circular
COLOUR	dark purple	white	yellow	white	white
MARGIN	entire	irregular	entire	entire	entire
ELEVATION	raised	raised	raised	raised	raised
OPACITY	opaque	opaque	opaque	opaque	opaque
SURFACE TEXTURE	smooth	mucoid	smooth	smooth	smooth
GRAM CHARACTER	G-ve, rods	G-ve, rods	G -ve, coccobacilli	G- ve, rods	G-coccobacillus

4.3 Biochemical tests:

Indole Test:

Table 4.3

Isolates from different agar.	image	result
TCBS agar Isolate 4		positive

ZMA Isolate 3		negative
EMB Isolate 1		negative

SS Isolate 2		positive
MAC Isolate 5		negative

Inference:

Certain bacteria produce enzyme tryptophanase that can metabolise tryptophan.

The indole reagent that aldehyde reacts with indole and produces distinctive colour. Hence pink to red colour rings are formed and it is referred to as a positive result. Some of the organisms that produce indole positives are *E.coli, V. cholerae, Enterococcus faecalis.* Etc,

Citrate Utilisation test:

Fig 4.1 citrate test showing positive and negative result

Table 4.4

Agar	Result
MAC isolate 5	negative
TCBS isolate 4	positive
ZMA isolate 3	negative
EMB isolate 1	positive
SS isolate 2	positive

Inference:

This test is to detect if the bacteria that was isolated can grow with citrate as the sole energy source. Examples of certain bacteria that give positive results are *Klebsiella spp., Salmonella spp., Enterobacter spp.*

Catalase Test:

Fig 4.2 catalase test showing positive results and negative results after addition of hydrogen peroxide

Table 4.5

Agar	Results
SS isolate 2	negative

ZMA isolate 3	negative
MAC isolate 5	positive
EMB isolate 1	negative
TCBS isolate 4	positive

Inference:

Microorganisms produce toxic by-products like hydrogen peroxide and superoxide radicals during metabolic activity. These can be toxic to the organism hence they produce enzyme catalases that hydrolyse hydrogen peroxide. It results in the release of air bubbles to the organism when hydrogen peroxide is added.

Biochemical test kit:

Fig 4.3 biochemical kits of HIMedia showing results after incubating for 18-24 hours

Table 4.6

media	SS	EMB	TCBS	ZMA	MAC
indole	+	-	+	-	-

MR	-	+	+	-	-
VP	-	-	-	-	-
citrate	+	+	-	+	-
glucose	+	+	+	+	-
adonitol	-	-	-	-	-
arabinose	-	-	-	-	-
lactose	-	-	-	-	-
sorbitol	-	-	+	-	-
mannitol	-	+	+	-	-
rhamnose	-	-	-	-	-
sucrose	-	-	+	-	-

Inference:

The biochemical test was carried out and from which the organism up till genus level was tried to be identified. But the results were inconclusive.

4.4 Antibiotic susceptibility test:

EMB Agar- Isolate 1:

Fig 4.4 zone of inhibition for antibiotics on MHA

Table 4.6

Antibiotic (HI Media G-IV-minus octa disc)	concentration of Antibiotic (mcg)	concentration of inoculum (microliter)	Zone of inhibition (mm)	susceptibility (zone of clearance)	susceptibility
A	10	0.2	-	-	Resistant
Co	25	0.2	-	-	Resistant
T	25	0.2	23mm	+++	Sensitive
SI	200	0.2	-	-	Resistant
S	10	0.2	23mm	+++	Sensitive
G	10	0.2	13mm	++	Resistant
Ci	25	0.2	9mm	++	Resistant

Ch	5		-	-	Resistant

Inference:

The negative sign represents that the organism is resistant to the antibiotic i.e., it can grow unaffected even in the presence of that particular antibiotic. The zone of inhibition refers that the antibiotic is inhibiting the growth of the microorganism surrounding the disc infused with the antibiotic. The isolate is resistant to antibiotics such as ampicillin and chloramphenicol, Co- trimoxazole, etc,.

SS Agar- Isolate 2:

Fig 4.5 zone of inhibition on MHA media shown for various antibiotic

Table 4.7

Antibiotic (HI Media G-VII- plus octa disc)	concentration of Antibiotic (mcg)	concentration of inoculum (microliter)	Zone of inhibition. (mm)	susceptibility (zone of clearance)	susceptibility
CH	30	0.2	-	-	Resistant
CX	5	0.2	-	-	Resistant
O	30	0.2	6mm	+	Resistant
CO	25	0.2	9mm	+	resistant
CD	2	0.2	-	-	Resistant
G	10	0.2	14mm	+++	Resistant

P	10	0.2	-	-	Resistant
E	15	0.2	-	-	Resistant

Inference:

This isolate is resistant to multiple antibiotics such as cephalothin, cloxacillin, penicillin etc,. The microbial resistance occurs when bacteria develop the ability to overcome the antibiotic that is specifically designed to kill them and survive even in the presence of that antibiotic.

TCBS plates- Isolate 4:

Fig 4.6 zone of inhibition on MHA media for various antibiotics

Table 4.7

Antibiotic (HIMedia OD037 octa disc)	concentration of Antibiotic (mcg)	concentration of inoculum (microliter)	Zone of inhibition (mm)	susceptibility (zone of clearance)	susceptibility
CO	25	0.2	14mm	+++	Resistant
E	15	0.2	13mm	++	Resistant
O	30	0.2	-	-	Resistant
G	10	0.2	14mm	+++	Resistant
CX	5	0.2	11mm	++	Resistant
CD	2	0.2	-	-	Resistant
CH	30	0.2	12mm	++	Resistant
P	10	0.2	-	-	Resistant

Inference:

The isolate that was inoculated showed sensitivity to oxytetracycline, Penicillin, Clindamycin, and other antibiotics that were introduced in the media. This isolate shows multidrug resistance. The bacteria could have developed antibiotic resistant genes and could also transfer it by plasmid mediated horizontal transfer.

MacConkey's Agar- Isolate 5:

Fig 4.7 zone of inhibition on MHA media

Table 4.8

Antibiotic (HIMedia OD037 octa disc)	concentration of Antibiotic (mcg)	concentration of inoculum (microliter)	Zone of inhibition (mm)	susceptibility (zone of clearance)	Susceptibility
CO	25	0.2	20mm	+++	Sensitive
E	15	0.2	-	-	Resistant
O	30	0.2	20mm	++	Sensitive
G	10	0.2	19mm	++	Sensitive
CX	5	0.2	11mm	+	Resistance
CD	2	0.2	11mm	++	Resistance
CH	30	0.2	19mm	++	Sensitive
P	10	0.2	-	-	Resistant

Inference:

The isolate is resistant erythromycin, penicillin and a

few other antibiotics. The resistance can be said to be the capability of microorganisms to survive high concentrations of antibiotics. There are several molecular strategies such as hydrolases or modified enzymes to deactivate the antibiotics. Etc.

ZMA- Isolate 3:

Fig 4.7 zone of inhibition on MHA media

Table 4.9

Antibiotic (HIMedia OD037 octa disc)	concentration of Antibiotic (mcg)	concentration of inoculum (microliter)	Zone of inhibition (mm)	susceptibility (zone of clearance)	susceptibility
CO	25	0.2	-	-	Resistant
E	15	0.2	15mm	+++	Sensitive
O	30	0.2	13mm	++	Resistant
G	10	0.2	14mm	++	Resistant
CX	5	0.2	8mm	+	Resistant
CD	2	0.2	17mm	+++	Sensitive
CH	30	0.2	8mm	+	Resistant
P	10	0.2	-	-	Resistant

Inference:

The isolate is resistant to co-trimoxazole, Penicillin and other antibiotics.. This can

occur as the microorganisms are adapting and developing some mechanism such as efflux pumps getting overactivated and decreased permeability which inturn decreases the accumulation of antibiotics.

4.5 Metal resistance:

TCBS: Isolate 4

HgCl2:

Fig 4.8 growth of organism on different concentration of the media

Table 4.10

metal concentration(mM)	growth of the isolate
0.1	-
0.2	-
0.4	-
0.5	-
1	-

Inference:

The organism is sensitive to the metal mercury, and could not grow in the presence of that metal in the media. Hence the plates were seen without any growth

CdSO4:

Fig 4.9 growth of microorganism on different concentration of metal concentration

Table 4.11

metal concentration(mM)	growth of the isolate
0.1	+++
0.2	++
0.4	+
0.5	++
1	-

Inference:

The organism can survive in the presence of the metal Cadmium in the media. It might be due to the various methods such as biofilm formation, Extracellular sequestration etc,. The MTC of the organism is 0.5 mM concentration and MIC is 1mM.

EMB: Isolate 1:

HgCl2:

Fig 4.10 growth of microorganism on different metal concentration

Table 4.12

metal concentration(mM)	growth of the isolate
0.1	-
0.2	-
0.4	-
0.5	-
1	-

Inference:

The microorganism inoculated with mercury in the media did not grow. It might be because it lacks a mechanism to survive in the presence of this metal.

CdSO4:

Fig 4.11 growth of microorganism on different concentration of metal

Table 4.13

metal concentration(mM)	growth of the isolate
0.1	+++
0.2	+++
0.4	++
0.5	++
1	+
1.2	-

Inference:

The organism grew in the presence of cadmium in the media. It might be due to various mechanisms such as intracellular sequestration, bioprecipitation etc, the MTC of the organism is 1mM and MIC is 1.2 mM.

SS agar: isolate 2:

CdSO4:

Fig 4.12 growth of microorganism on different concentration of metal

Table 4.14

metal concentration(mM)	growth of the isolate
0.1	+++
0.2	++
0.4	+
0.5	+
1	-

Inference:

The microorganism grew with the presence of cadmium in the media. The process of biotransformation and biofilm formation may be the reason for the survival of the microorganism in the presence of heavy metal. MTC of the organism is 0.5mM and MIC is 1mM

HgCl2:

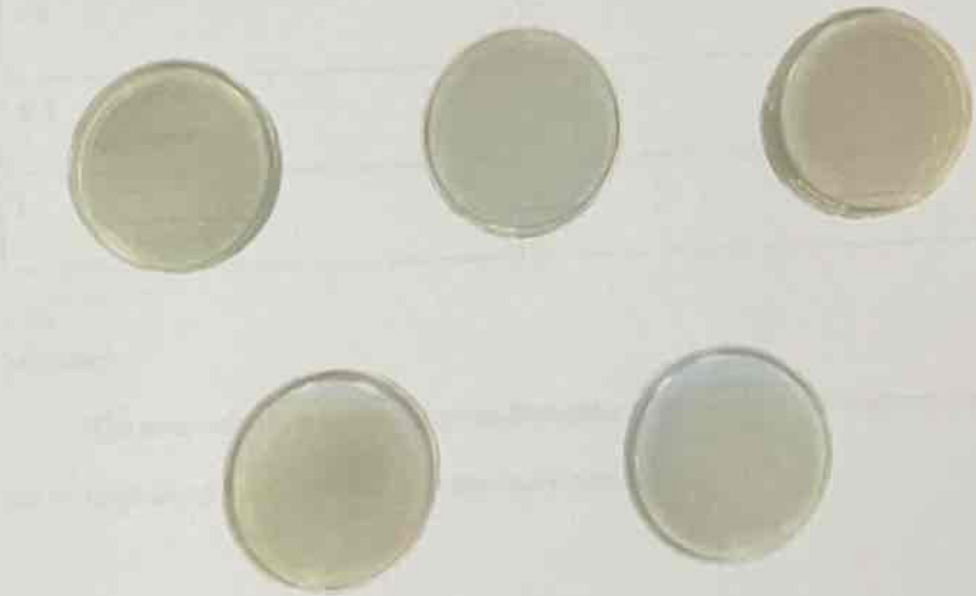

Fig 4.13 growth of microorganism on different concentration of metal

Table 4.15

metal concentration(mM)	growth of the isolate
0.1	-
0.2	-
0.4	-
0.5	-
1	-

Inference:

The microorganism did not grow on the media in the presence of mercury since it had no mechanism for survival against the heavy metal.

MacConkey agar: Isolate 5:

CdSO4:

Fig 4.14 growth of microorganism on different concentration of metal

Table 4.15

metal concentration(mM)	growth of the isolate
0.1	-
0.2	-
0.4	-
0.5	-
1	-

Inference:

The microorganism did not grow in the presence of cadmium in the media since it had no mechanism for resistance against the heavy metal.

HgCl2

Fig 4.15 growth of microorganism on different concentration of metal

Table 4.16

metal concentration(mM)	growth of the isolate
0.1	+++
0.2	-
0.4	-
0.5	-
1	-

Inference:

The organism grew in the presence of the mercury in the media since it might have had mechanism such as reduced intake of mercuric ion, extracellular sequestration of methylmercury, intracellular bioaccumulation of mercury, demethylation of mercury by organomercurial lyases etc, the MTC of the microorganism is 0.1 mM and MIC is 0.2 mM.

ZM agar:

CdSO4:

Fig 4.17 growth of microorganism on different concentration of metal

Table 4.18

metal concentration(mM)	growth of the isolate
0.1	+++

0.2	+
0.4	-
0.5	-
1	-

Inference:

The organism grew in the presence of the metal cadmium. This may be due to the presence of various mechanisms such as intracellular sequestration, efflux mechanism, biofilm formation etc,.. MTC of the organism is 0.2 mM and MIC is 0.4mM.

HgCl2

Fig 4.17 growth of microorganism on different concentration of metal

Table 4.18

metal concentration(mM)	growth of the isolate
0.1	-
0.2	-

0.4	-
0.5	-
1	-

Inference:

The organism did not grow in the presence of mercury since it might not have the mechanism to survive in the presence of mercury.

Plasmid isolation:

Fig. 4.18. Plasmid isolation was carried out and the agarose gel gel showed that there is no plasmid present. The organism was grown in Nutrient broth without the metal.

Inference:

The samples (isolates) were run with a standard of 1kb ladder for interpretation.

The plasmid was absent in the Isolates: 1,2,3,4,5. This may be due to the absence of plasmid in the microorganism or due to any error during the extraction.

Fig.4.19 : the plasmid isolation was carried out and agarose gel did not show any band that indicates the presence of plasmid. The organisms were grown in the presence of heavy metal.

Inference:

The plasmid was not found in the agarose gel electrophoresis against the 100kb ladder because the antibiotic and metal resistance conferring gene bands may be found in the

genome of the bacteria and they might lack plasmid. It may also be due to some error during the running through the gel.

Conclusion:

Various tests were carried out from the Bacteria that were isolated from the microplastic that was collected from marine environment (Cacra Beach). The isolates were tested for antibiotic and metal resistance in particular since marine environment is polluted with heavy metals, microplastics, other pollutants. The experiments were conducted that revealed that all the isolates (isolate 1,2,3,4,5) were showing both resistance (Co- Selection). This phenomenon may be due to the Co- Selection i.e, Co-Resistance or Cross- Resistance. This study is essential because the fishes in the marine environment ultimately reach humans since they consume the fishes. The MultiDrug Resistance organism also is transferred from fishes to the humans due to the consumption. The fishes also consume the microplastics thinking them as their food and it also reaches the humans and they remain the system. These ultimately lead to various health issues, and lead to something Fatal. The increase in the plastic consumption is alarming and due to which the threat to not only humans but also the Ecosystem is increasing rapidly.

APPENDIX

Media composition:

EMB:

Peptic digest of animal tissue	10g
Dipotassium phosphate.	2g
Lactose.	5g
Sucrose.	5g
Eosin.	0.4g
Methylene Blue.	0.06g
Agar.	13.5g
pH.	7.2

TCBS:

Yeast extract.	5g
Peptone.	10g

Sodium thiosulphate.	10g
Sodium citrate.	10g
Ox bile.	5g
Sucrose.	20g
Sodium chloride.	10g
Iron (III) citrate.	1g
Bromothymol blue.	0.04g
Thymol blue.	0.04g
Agar.	15g
Distilled water.	1000ml

ZMA:

Meat peptone	5g
Yeast extract	1g
Iron citrate	0.1g
Sodium chloride	19.45g
Sodium sulphate	3.24g
Sodium bicarbonate	0.16g

Sodium silicate	0.004g
Sodium fluoride	0.0024g
Disodium phosphate	0.008g
Calcium chloride	1.8g
Magnesium chloride	8.8g
Potassium chloride	0.55g
Potassium bromide	0.08g
Strontium chloride	0.034g
Ammonium nitrate	0.0016g
Boric acid	0.02g
Agar	15.0g
Distilled water	1000ml

Final pH 7,6 ± 0,2 at 25°C

SS:

Peptone.	500mg
Beef extract.	500mg
Na- citrate.	850mg

Sodium thiosulphate.	850mg
Fe-citrate.	100mg
Distilled water.	90 ml
Ph.	7.0

Brilliant green.	1 crystal
Neutral red.	2mg
Bile salt.	0.85g
Agar.	3g
Lactose.	3g
Distilled water.	10ml

MAC:

Peptone.	2g
Na- taurocholate.	0.5g
2%(w/v) neutral red (40mg in 50% ethanol).	0.7ml
NaCl.	0.5g
Distilled water.	80 ml
PH.	7.4

Agar.	3g
Lactose.	1g
Distilled water.	100 ml

MSA:

Protease peptone.	10g
Beef extract.	1g
NaCl	7.5g
D- Mannitol.	10g
Phenol Red.	0.025g
Agar.	15g
pH.	7.2

Nutrient agar:

Peptone	1g
Beef extract.	0.3g
NaCl.	0.5g

Distilled water.	100ml
pH.	7.5
Agar.	3g

Muller Hinton Agar:

Beef Extract	2g
Acid Hydrolysate of Casein	17.5g
Starch	1.5g
Agar	17g
Distilled water	1000ml

Final pH 7.3 ± 0.1 at 25°C

TBE Buffer:

tris base.	108 g
boric acid.	55 g
double-distilled H_2O.	900 ml
0.5 M EDTA solution	40 ml

(pH 8.0)

ABBREVIATIONS

Media:

ZMA- Zobell Marine Agar

EMB- Eosin methylene Blue Agar

TCBS- Thiosulfate Citrate Bile salt Sucrose agar

SS- salmonella Shigella agar

MAC- Mac Conkey's agar

Results:

Biochemical test kit:

+- positive

— - negative

Antibiotic resistance:

+++ - zone more than _ mm

++ - zone more than

+ - zone more than

— - no zone

Metal resistance:

+++ - growth of the isolate till 3 rd. quadrant streak

++ - growth till 2nd quadrant

+ - growth only in inoculation zone

— - no growth

Antibiotics:

Ch- Cephalothin

Cx- Cloxacillin

O- Oxytetracycline

Co- Co- Trimoxazole

Cd- Clindamycin

G- Gentamicin

P- Penicillin-G

E- Erythromycin

Cx- Cefoxitin

C- Chloramphenicol

A- Ampicillin

S- Spectinomycin

S3- Sulphatriad

Ci- Ciprofloxacin

T- Tetracycline

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