Toxicity Status Of Whiteleg Shrimp (*Litopenaeus vannamei*) From Sluice Gates Harvest Sites In North Goa (Saint Estevam Island, Cundaim & Madkaim)

By

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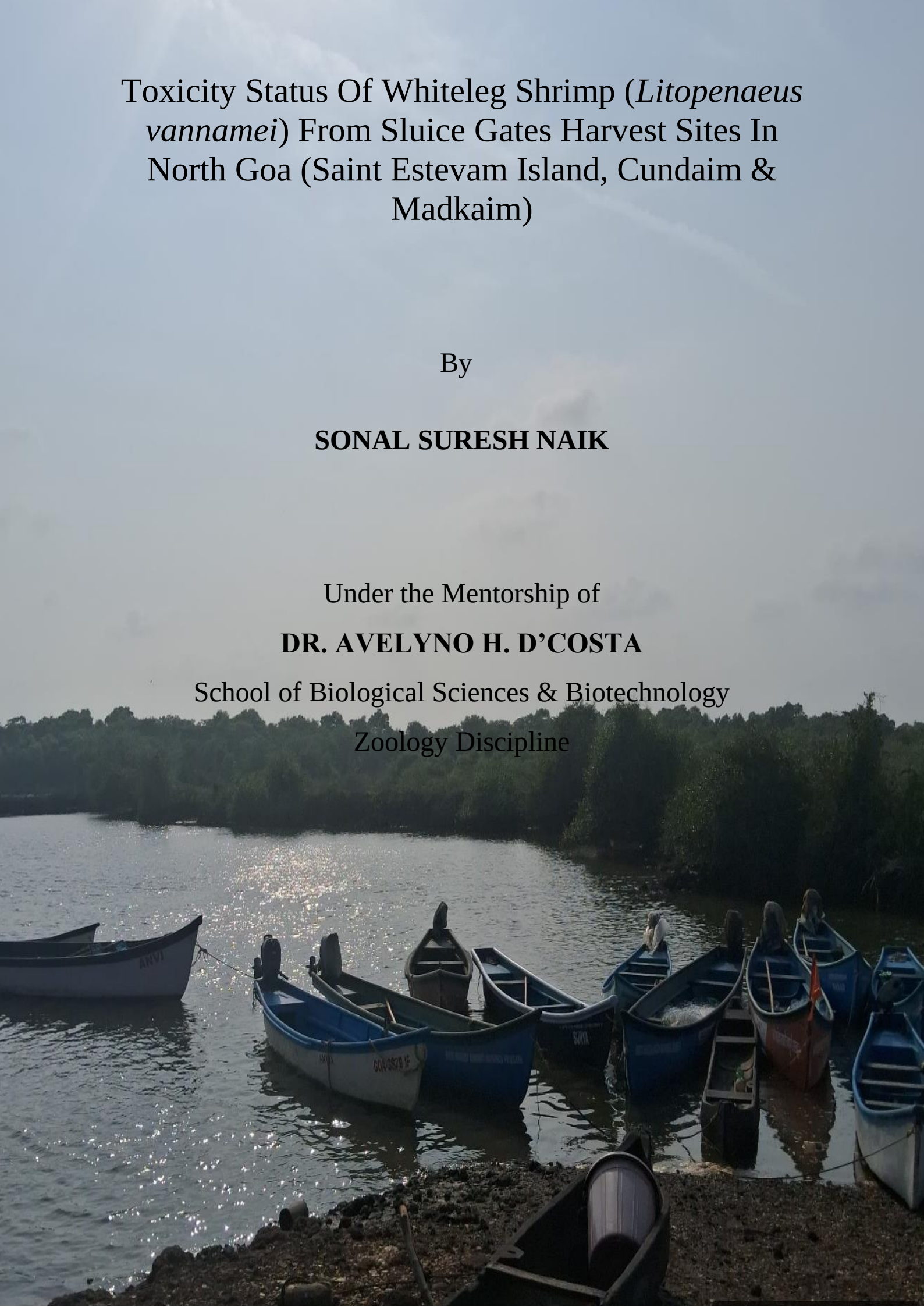

Toxicity Status Of Whiteleg Shrimp (*Litopenaeus vannamei*) From Sluice Gates Harvest Sites In North Goa (Saint Estevam Island, Cundaim & Madkaim)

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DECLARATION BY STUDENT

I hereby declare that the data presented in this Internship report entitled, "Toxicity Status Of Whiteleg Shrimp (*Litopenaeus vannamei*) From Sluice Gates Harvest Sites In North Goa (Saint Estevam Island, Cundaim & Madkaim)" is based on the results of investigations carried out by me in the Zoology Discipline at the School of Biological Sciences & Biotechnology, Goa University, under the mentorship of Dr. Aveyleno. H. D'Costa, and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will not be responsible for the correctness of observations / experimental or other findings given the Dissertation report/work.

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COMPLETION CERTIFICATE

This is to certify that the Dissertation report "Toxicity Status Of Whiteleg Shrimp (Litopenaeus vannamei) From Sluice Gates Harvest Sites In North Goa (Saint Estevam Island, Cundaim & Madkaim) is a bonafide work carried out by **Ms. Sonal Suresh Naik** under my mentorship in partial fulfilment of the requirements of ZOO-651 Dissertation (16 credits) course in the Discipline of Zoology at the School of Biological Sciences & Biotechnology, Goa University.

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PREFACE

Microplastics, ubiquitous in our environment, have raised significant concerns due to their potential adverse effects on ecosystems and human health. Beyond their physical impact, microplastics pose a significant threat through chemical contamination. These particles have the ability to adsorb and concentrate various toxic compounds from the surrounding environment.

Microplastics can undergo bioaccumulation within aquatic organisms, resulting in increased concentrations as they move up the food chain. This phenomenon, coupled with biomagnification, whereby contaminants become more concentrated at higher trophic levels, poses a significant risk to predators, including humans, at the top of the food chain. Detecting and validating the presence of microplastics, particularly in aquatic organisms like shrimps, is crucial for understanding the extent of this environmental challenge.

Validating the presence of plastic pollution and assessing its effects on genetic, biochemical, and physiological levels can provide valuable insights into the severity of the problem and help in formulating effective mitigation strategies. By examining genotoxicity, researchers can evaluate the potential harm plastic pollution may cause at the genetic level, including DNA damage and mutation. Biochemical and physiological assessments can further elucidate how plastic pollution affects the health and functioning of organisms in these ecosystems. They can serve as a basis for implementing measures to reduce plastic usage, improve waste management practices, and protect the marine environment.

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LIST OF ABBREVIATIONS

- µ- Raman- Micro Raman Spectrophotometer
- µm - Micrometer
- ALP- Alkaline Phosphatase
- ALT- Alanine Transferase
- CAT – Catalase
- Cm- centimeteres
- CMPI- Coefficient of Microplastic Impact
- EDTA- Ethyelene diamainetetra acetic acid
- g- gram
- Kg- Kilogram
- ml- Milli Meter
- MP- Microplastic
- OD – Optical Density
- Protein H – Protein of Hepatopancreas
- Protein M- Protein of Muscle
- TBARS- Thiobarbituric Acid Reactive Substances

ABSTRACT

The study investigated the abundance, characteristics, and impact of microplastics in shrimp tissues and water samples collected from sluice gate harvest sites in Goa over a three-month period. Microplastics were found to accumulate in the hepatopancreas of shrimps and in water samples, with Saint Estevam Island exhibiting the highest concentration. Various shapes and sizes of microplastics were identified, with fibers and fragments being prevalent. Microplastic colors varied, with blue being dominant. Polymer analysis revealed the presence of several types, confirmed by Micro-Raman Spectrophotometer. The Coefficient of Microplastic Impact (CMPI) assessment highlighted moderate to substantial impacts, particularly from fibers. The study further investigated genotoxicity through comet assays, revealing the absence of DNA damage in shrimp tissues from all three sites.

Biochemical estimations were conducted to assess protein concentration, catalase enzyme activity, lipid peroxidation levels, alanine transferase (ALT), alkaline phosphatase (ALP), and condition index. Significant differences were observed across months and sites for all biochemical parameters, indicating variations in physiological responses to microplastic exposure. Pearson's correlation tests revealed associations between biochemical parameters, highlighting potential interactions and implications for shrimp health. These findings underscore the pervasive nature of microplastic pollution in aquatic environments and emphasize the importance of continued monitoring and mitigation efforts to safeguard ecosystem health and food safety.

Keywords*:- Microplastic, Sluice gate, Biomarkers, Food Safety, Bioaccumulation*

CHAPTER 1

INTRODUCTION

1.1 Plastic pollution in the environment

From land to sea, plastic pollution pervades: terrestrial environments, shorelines, oceans, and even deep sea depths (Barnes et al., 2009). Plastics originate from elongated chains of polymeric molecules derived from both organic and inorganic raw materials, including carbon, silicon, hydrogen, oxygen, and chloride. Typically sourced from oil, coal, and natural gas, these materials serve as the primary constituents for plastic production (Shah et al., 2008).

Plastics are versatile materials that can be moulded into a diverse array of products for various applications. Known for their durability, affordability, and lightweight nature, plastics play an integral role in numerous industries, including food packaging, aerospace, automotive, engineering, medical, and construction. With economic growth driving increased demand, the global manufacturing and utilization of plastics have experienced rapid expansion over recent decades (Hopewell et al., 2009).

Plastic pollution is the act of introducing plastics, irrespective of their sizes, shapes, or types, into the environment. This intrusion poses potential threats to the ecosystem, organisms within it, and potentially human health (Li et al., 2021). When plastics enter natural ecosystems, they may be carried from land to rivers, eventually finding their way into the ocean (Jambeck et al., 2015). The amount of plastic in the ocean is steadily rising each year, with a fraction of it breaking down into microplastics. Such MPs could be ingested by a wide variety of organisms (Ugwu et al. 2021). Marine organisms are valuable bioindicators of aquatic system health, reflecting the presence and impact of diverse environmental pollutants (Lopes et al., 2001).

1.2 Microplastic and its impact on the ecosystem

The ubiquitous man-made contaminant known as microplastics (Phuong et al., 2016). Plastics are categorized into four size groups: (>1m) megaplastic, (<1m) macroplastic, (<2.5cm) mesoplastic and (<5mm) microplastic (Wang et al., 2018). Microplastics are defined as tiny pieces of plastic measuring less than 5 mm in size. They can originate from various sources, including the direct spillage of plastic particles and the breakdown of larger plastic items (Moore, 2008; Moore et al., 2011).

MPs are classified into two types: primary MPs that are manufactured in microscopic sizes and secondary MPs generated from the fragmentation of larger debris (Auta et al., 2017; Barnes et al., 2009). Large plastic breaks down to small size debris due to physical, chemical and biological factors such as the UV radiation, mechanical abrasion and biological degradation (Andrady, 2017; Thompson et al., 2004; Wang et al., 2018). Natural phenomena like hydrodynamics, wind, and ocean currents play a significant role in dispersing microplastics throughout the marine environment (Martin et al., 2017).

Marine organisms are susceptible to ingestion and entanglement in microplastics due to their small size, leading to suffocation, starvation, and chemical harm. These risks present significant and increasing threats to the diversity of marine life and the overall health of aquatic ecosystems (Wang et al., 2018).

1.3 The effects of microplastics and its accumulation in aquatic environments

Bioindicators are essential for evaluating environmental pollution levels, particularly in areas under anthropogenic pressure (D'Costa et al., 2018). The behavior and destiny of marine microplastic particles are determined by their physical attributes, including density,

size, and shape (Chubarenko et al., 2016). Microplastics have been observed across diverse marine habitats of the open oceans, enclosed seas, beaches, surface waters, the water column, and the deep seafloor (Lusher, 2015). Once microplastics are accumulated, toxicity could also arise from leaching constituent contaminants (Wright et al., 2013).

Prior to comprehending the fate of microplastics, it is essential to elucidate their transport and distribution mechanisms, along with assessing their potential physical and chemical impacts on marine organisms (Lusher, 2015). Microplastic ingestion by invertebrates represents a critical, yet understudied, pathway for plastic entry into aquatic food webs. Elucidating this process is vital for understanding the ultimate fate and potential human health risks associated with microplastic contamination in seafood (Montague and Busch, 2020).

Microplastic debris pervades every marine habitat, posing a ubiquitous threat (Sul and Costa, 2014). Many marine organisms have been observed to ingest microplastics including Zooplanktons (Cole et al., 2013); Barnacles (Xu et al., 2020), Bivalves (Ding et al., 2021; Ding et al., 2018), Crustaceans (D'Costa 2022); Corals (Soares et al., 2020) Echinoderms (Plee and Pomory 2020); Sea Birds (Weitzel et al., 2021); Fishes (Compa et al., 2024); sea turtles and Marine Mammals (Meaza et al., 2021).

Microplastics, either independently or in conjunction with hazardous contaminants, possess the capacity to undergo transfer along trophic levels via bioaccumulation and biomagnification mechanisms. Consequently, this phenomenon culminates in the contamination of both biotic and abiotic marine resources, thereby posing potential hazards to human health. Once introduced into the environment, degradation of plastic

takes place, breaking down into diverse forms, such as fibers, fragments, films, and pellets (Chubarenko et al., 2016; Gurjar et al., 2022).

1.4 Detection and identification of microplastics

The shape type, size, abundance, spatial distribution, polymer composition and surface morphology of the microplastics can be identified by a range of advanced microscopic and micro-analytical methods (Zhou et al., 2018). Further analysis is crucial for confirming the identity of microplastic particles and gaining insights into their plastic type, potential origin, and source (Zantis et al., 2021).

Biota-derived microplastics are typically obtained through organism dissection, with organs like livers, gills, and guts separated. Density separation effectively isolates microplastics, while chemical digestion removes organic material. Enhancing polymer identification, visual sorting is combined with chemical analysis, utilizing pyrolysis, gas chromatography, mass spectrometry, Fourier-transform infrared spectroscopy, and Raman spectroscopy (Mai et al., 2018).

Raman spectroscopy stands out as the leading non-destructive technique for identifying microplastics, particularly those smaller than 20 μm (micrometers). Challenges such as weak signals and fluorescence interference can be mitigated through meticulous cleaning protocols, advanced baseline removal algorithms, and the utilization of improved detectors (Araujo et al., 2018).

Microplastics found in aquatic environments come in various polymer types, including Polyethylene (PE), Polyethylene terephthalate (PET), Polyamide (PA), Polypropylene

(PP), Polystyrene (PS), Polyvinyl Alcohol (PVA), PolyVinyl Chloride (PVC), Acrylate, High-Density Polyethylene (HDPE), Low-Density Polyethylene (LDPE), Polyurethane (PUR), Polycarbonate, Epoxy, Polytetra Fluoroethylene etc (Ashrafy et al., 2023).

1.5 Human health implications of microplastic exposure

Microplastics (MPs) represent a widespread environmental issue and posing significant threats to human and animal health. Studies indicate potential harm through inhalation, ingestion, and dermal contact, leading to inflammation, oxidative stress, and even genotoxicity (Vethaak and Legler, 2021). MPs can potentially be passed down the food chain to humans (Renura et al., 2022).

MPs have been identified in diverse human organs, underscoring their pervasive presence in the human body. Studies report their detection in crucial organs such as the placenta and liver, as well as bodily fluids like sputum, breast milk, and blood samples (Barcelo et al., 2023).

Chemicals in plastic production are toxic, detected widely in human tissues via biomonitoring, signifying substantial exposure. Animal studies emphasize health risks, urging stringent regulations and ongoing research to mitigate harm (Talsness et al., 2009). Furthermore, the ingestion of microplastics through seafood consumption can lead to health complications such as inflammation, cell proliferation, necrosis, and impaired immune system function (Smith et al., 2018).

1.6 Strategies to mitigate microplastic pollution

Plastics offer benefits, but current production, use, and disposal are unsustainable, posing risks to wildlife and human health. Addressing these issues demands collaborative efforts from individuals, industries, and policymakers, to tackle environmental hazards to ongoing plastic production expansion (Thompson et al., 2009).

Smith et al., (2018) reported that human activities have resulted in widespread microplastic contamination in marine environments, leading to ingestion by various wildlife species, including fish and shellfish. They stress the urgent need for research to define the toxicity of microplastics, address knowledge gaps and formulate the mitigation strategies, recognizing the critical importance of seafood consumption for human nutrition.

Goa, positioned on the central west coast of India, is renowned as a top international tourist hotspot but is also known for harboring the highest levels of beach litter and plastic waste compared to other coastal states in the country (Nigam et al., 2022). Goa's dynamic coastline, influenced by reversing monsoon winds and traversed by rivers draining urban and industrial areas, experiences significant plastic pollution. This threatens tourism, a vital economic sector, by degrading scenic beaches and ecosystems (Veerasingam et al., 2016).

Shrimps serve as effective indicators for assessing the quality of aquatic systems exposed to various pollutants (Duarte-Restrepo et al., 2020). Despite its popularity in Goa, the safety of *Litopenaeus vannamei* consumption remains unclear due to the lack of toxicity studies. Furthermore, no studies have been conducted at the selected sites to assess the

toxicity status of *Litopenaeus vannamei*. Therefore, studying microplastics in invertebrates is crucial to comprehending how microplastics enter aquatic food webs.

Thus this study aims to detect microplastic accumulation in water and shrimps from sluice gate harvest sites (Saint Estevam island, Cundaim and Madkaim), to evaluate the genotoxicity prevailing in shrimps and to analyse the physiological and biochemical parameters in correlation with accumulation of microplastic in shrimps.

CHAPTER 2

REVIEW OF LITERATURE

In a review by Thompson et al., (2009), the authors discuss the widespread use of plastics and its benefits to society. However, they also raise concerns about the environmental impact of plastic production, disposal, and its potential harm to wildlife. The authors conclude by highlighting the need for sustainable solutions such as recycling, bioplastics, and reduced plastic use.

D'Costa et al. (2018) emphasize the essentiality of regular estuarine monitoring to identify xenobiotic pollutants. Wright et al. (2018) emphasize the significance of ongoing research to comprehend the environmental impacts of microplastic accumulation, which has evolved into a new marine habitat. They stress the necessity for developing effective methods to control marine litter in light of this emerging issue.

Mai et al. (2018), addresses the growing concern over microplastic (MP) pollution in aquatic environments, highlighting the need for standardized methods in MP research. Analytical techniques for sampling, identification, and quantitation are evaluated, with prevalent methods. Density separation and chemical digestion aid in isolating MPs, while spectroscopic methods are key for identification.

Liu et al. (2020) highlighted the global concern over the widespread distribution of microplastic (MP) pollution, particularly emphasizing the impact of weathering on its physicochemical properties and interactions with pollutants. They examined changes in MP physicochemical properties and the release of additives and intermediates during weathering, affecting sorption behavior for environmental pollutants.

Talsness et al., (2009) reviewed the toxicological implications of chemicals in plastic production, emphasizing their potential harm to human health. Biomonitoring data presented underscores the widespread presence of these chemicals in human tissues, indicating

substantial exposure from diverse sources. The review emphasizes the imperative for continued research to mitigate risks posed by plastic.

Lusher (2015), states that the size of microplastics influences their impact on organisms, highlighting that smaller sizes have more significant effects at the cellular level. Micrometer-sized plastics are readily ingested and expelled, whereas nanometer-sized ones can traverse cell membranes. Evidence suggests microplastics enter food chains and there is trophic transfer between predators and prey. Emphasizing the necessity for additional research across a range of marine organisms to fully understand the environmental implications of microplastics.

Microplastics, such as fine particulate air pollution, can enter the human body via inhalation and ingestion, potentially leading to health issues. This comparison highlights the need for further investigation into the health risks associated with microplastic exposure (Vethaak and Legler, 2021).

In their study, Gupta et al. (2021) explored microplastic (MP) variations across seasons in Goa's Mandovi-Zuari estuarine system. They found higher MP concentrations during the wet season, attributed to increased freshwater influx carrying terrestrial plastic debris. Mismanaged sewage treatment plants, washing machine effluents, and urban and industrial waste were identified as primary sources, emphasizing the need for mitigation measures.

Castaneda et al. (2022) had identified and characterized microplastics in the gastrointestinal tract, gills, and exoskeleton of *Litopenaeus vannamei* in a coastal lagoon from the SE Gulf of California.

Daniel et al. (2020) underscored the growing concern about microplastic contamination in seafood, particularly its implications for food safety and human health. Their study on *Fenneropenaeus indicus,* reveals the presence and seasonal fluctuation of microplastics in soft tissues. The research identifies 128 microplastics, primarily fibers, signifying a notable

increase in microplastic presence, especially during the monsoon season. These findings emphasize the potential human exposure to microplastics via shrimp consumption, urging further investigation and mitigation efforts.

Maharana et al. (2020) found a notable prevalence of plastic fragments and pellets, mainly white-colored polyethylene polymers, on west coast beaches of India. Their study on *Litopenaeus vannamei* revealed physiological changes, including increased lipid peroxidation (LPX) levels measured by TBA assay, alongside a slight elevation in antioxidants like catalase (CAT) and glutathione-S-transferase (GST) at higher doses of polyethylene microbeads, indicating a response to ROS production.

In another study *Litopenaeus vannamei* were exposed to varying MP concentrations for 48 hours and they showed differential survival rates and immune-related gene expression. Results indicate changes in survival rates, immune gene expression, and microbial composition, particularly under high MP exposure, potentially leading to shrimp mortality, thus contributing to the effects of MP on shrimp and their microflora (Wang et al., 2021).

Hsieh et al. (2021) showed the effect of polyethylene MPs in *Litopenaeus vannamei* which influenced antioxidant enzymes, increased lipid peroxides, and caused tissue damage. Exposure to microplastic can seriously affect shrimp gills, causing physical harm to the gill structure, decreased oxygen intake, and an increased risk of infection.

Saha et al. (2021) conducted the first assessment of microplastics (MPs) in Goa's Sal estuary across water, sediments, and biota. They found that MPs in shellfish and finfish species closely resembled those in the surrounding environment, predominantly consisting of colored fibers.

Gurjar et al. (2021) conducted a comprehensive review on the accumulation of microplastics, results confirms the existence of microplastics in shrimp habitats extending beyond coastal regions, demonstrating that shrimps harvested from these coastal fishing grounds harbor

microplastics within gastrointestinal tracts of *Metapenaeus monoceros*, *Parapeneopsis stylifera*, and *Penaeus indicus.*

Han et al. (2022) found that exposing Whiteleg shrimp to both microplastics and bisphenol A (BPA) resulted in more severe consequences than exposure to either substance individually. The combination led to more BPA building up in the shrimp's reproductive organs (testis and ovaries).

Reunura and Prommi (2022), indicated that consuming shrimps and prawns without first removing the MPs from their GTs is one of the means by which humans get exposed to MPs.Yoon et al. (2022) concluded that microplastic pollution significantly impacts marine organisms like *Litopenaeus vannamei*, causing digestive system damage and hindering growth and fertility.

Gupta et al., (2024) conducted a comparative assessment of microplastics (MPs) in Goa's major rivers before and after the COVID-19 pandemic. They found a significant decrease in MP concentrations post-pandemic. Dominant polymer types shifted, indicating changes in plastic sources. Overall, reduced anthropogenic activities during the pandemic period led to decreased plastic waste and improved water quality in coastal areas of Goa, highlighting the pandemic's impact on plastic pollution dynamics.

Kalangutkar et al. (2024) investigated microplastics in Goa's Chapora River, finding moderate levels (0.25 particles/L) with fibers being the most common shape and PET the dominant polymer. This study provides valuable baseline data for managing future plastic pollution in Goan rivers.

CHAPTER 3

MATERIALS & METHODS

3.1 STUDY AREA

Goa, with its diverse ecosystems and rich biodiversity, serves as an ideal study area for toxicological research. Goa encompasses an area of 3,702 km. It lies between the latitudes 14°53′54″ N and 15°40′00″ N and longitudes 73°40′33″ E and 74°20′13″ E. The estuarine environment of Goa, presents a unique opportunity for toxicological studies due to its specific ecological characteristics. Its mix of urban areas, industrial zones, and natural habitats offers insights into the impact of environmental contaminants on both terrestrial and aquatic ecosystems.

Sluice gates or "*Manos"*, control water flow between reservoirs and estuaries. They open and close automatically with tides, during high tide shutters close to regulate water intake, and during low tide, they automatically release excess field water into the estuary. Bag nets, known as "*manxeche jale*," are employed at the sluice gate to harvest the fishes.

The three study sites (plate 1, fig 2) are distributed across the riverine network of Mandovi and Zuari river are as follows:

3. 1.1 SAINT ESTEVAM ISLAND

St. Estevam Island / Juvem Village is located in Tiswadi taluka, North Goa (plate 1, fig 3). The study site at sluice gate is called as "*Tarzo manos*". It is situated along the Mandovi River in the North Goa district of Goa. It lies between latitude 15°31'40"N and longitude 73°55'59"E. A variety of fishing techniques are employed, ranging from traditional dugout canoes to modern plank-built boats. Fishermen utilize an array of fishing gears like bag nets, hand nets, seine nets, and gill nets of different mesh sizes to catch a diverse range. The island is surrounded by lush mangrove vegetation, providing a rich ecosystem for crustaceans such as prawns and shrimps, as well as fish species like grey mullet, lady fish, snappers etc.

3. 1.2 CUNDAIM

Cundaim / Kundaim village is located in Ponda Taluka, Goa (plate 1, fig 4). The study site at sluice gate is called "*Chikalpaine manos*". It lies between latitude 15°26'46"N and longitude 73°56'49"E. It is located along the Cumbharjua Canal which connects the Mandovi river network to the Zuari river network. Cundaim village has a unique blend of urban development, industrial activities, and proximity to natural habitats. Fishermen employ a variety of fishing methods, fishing crafts such as dugout canoe. They utilize various fishing gears such as bag nets, hand nets, and gill nets of different mesh sizes to harvest the seafood. The area is surrounded by lush mangrove vegetation, creating a thriving ecosystem for crustaceans like prawns, shrimps, and crab, molluscs such as edible oysters as well as an abundance of fish species including grey mullet, snappers, Asian sea bass, tilapia, tiger perch, Indian anchovy, Pearls spot, etc.

3. 1.3 MADKAIM

Madkaim / Marcaim Village is located in Ponda taluka, Goa (plate 1, fig 5). The study site at sluice gate is called as "*Karyachi manos*". It lies between latitude 15°25'34"N and longitude 73°55'51"E. It is located along the river Zuari in the South Goa district of Goa. Fishermen use a variety of vessels and nets for their fishing operations. The flourishing mangrove environment supports an abundance of species, from prawns, clams and crabs to grey mullet, snappers, Asian sea bass, Indian anchovy, Pearlspot, Silver biddy, and many more. Additionally, the area supports the cultivation of green mussels contributing to the local economy and food supply.

3.2 SAMPLING METHOD

The study was conducted from January to March 2024, during which water samples (500ml from each site) and shrimps (30 from each site) were collected monthly from the three sluice gates at Saint Estevam Island, Cundaim, and Madkaim. Ethical approval for animal research was obtained prior to conducting the study from the Institutional Animal Ethics Committee (IAEC), with the approval reference number GUZ/IAEC/23-24/N18. Harvesting at the sluice gates was done during low tide, when water levels were at their lowest. Live samples were directly transported from the sites to the laboratory in aerated bags for further analysis. Haemolymph was extracted from live shrimps and were then stored in the deep freezer (-20^{0}C) till the further analysis.

3.3 STUDY ANIMAL

Whiteleg Shrimp (*Penaeus vannamei),* Boone, 1931

Penaeus vannamei is distributed along the East and West Coast of India (plate 1, fig 1). Identification was done by using manual on "Taxonomy and identification on commercially important crustaceans of India" by Jose et al., (2013). This shrimp features movable chelae on its first three pairs of walking legs and a distinct third thoracic segment. With a moderately long rostrum bearing 7-10 dorsal and 2-4 ventral teeth, its color is translucent with dusky bands and white legs. Pronounced antennal and hepatic spines are present, and its last abdominal segment exhibits three lateral ridges. Adult *Penaeus vannamei* spawn in the ocean, releasing eggs that hatch into non-feeding larvae. These larvae progress through stages of zoea, mysis, and postlarva before migrating to estuaries as juveniles, while adults return to the sea for spawning (Food and Agricultural Organization).

Classification:-

Kingdom: Animalia

Phylum: Arthropoda

Subphylum: Crustacea

Class: Malacostraca

Order: Decapoda

Family: Penaeidae

Genus: Penaeus

Species: *Penaeus vannamei*

Local Name :- "*Sungata*"

3.4 QUALITY ASSURANCE

All experiments were conducted in sterile conditions wearing clean lab coats. Glassware was cleaned with double distilled water, sterilized, and heated in an oven to prevent contamination. Filter papers were carefully handled so as to minimize exposure to air and placed in clean Petri dishes so as to prevent contamination of extracted microplastics. Samples were consistently covered with aluminium foil, and all chemicals used were of analytical grade from Himedia, India and SD Fine, India.

3.5 MICROPLASTIC ANALYSIS

3.5.1 Extraction of Microplastic from water sample (Luo et al., 2018)

Principle:-

The digestion of water samples with 30% hydrogen peroxide facilitates the breakdown of organic matter, enabling the extraction of microplastics by oxidizing the organic matrix while maintaining the integrity of the plastic particles.

Materials Required:-

Beakers, Conical flasks, measuring cylinder, water samples (from sites), Aluminium foil, Membrane filter paper (0.22µm), Incubator, Hot air oven and Vaccum filtration pump.

Chemical Required:-

30% H_2O_2

Procedure:-

250 ml of sample water was taken then approximately 200-250 mL H_2O_2 (30%) solution was added to each 500ml of conical flask to dissolve the organic matter of the surface water in conical flask. The conical flasks were covered with aluminium foil and placed in an incubator at 65^0C for approximately 24-48 h (depending upon the dissolution level). After the dissolution process, samples were filtered with the 0.22µm size filter paper, then filter paper was dried in a hot air oven at 40^0C and the filters were stored in dry Petri dishes for further observation. The protocol was done with the slight modifications in the method given by Luo et al., (2018).

3.5.2 Extraction of Microplastic from tissue sample *(*Dehaut et al., 2016*)*

Principle :-

A two-step process efficiently digests biological material for analysis, initial treatment with 10% KOH solution, followed by filtration, breaks down organic matter. Persistent residues undergo further oxidation with 30% H_2O_2, ensuring complete degradation before subsequent analysis.

Materials Required:-

Beakers, Conical flasks, measuring cylinder, sample, Aluminium foil, 10% KOH, 30% H_20_2, Membrane filter paper (0.22µm), Incubator, Hot air oven and Vaccum filtration pump.

Chemical required:-

30% H_2O_2

10% KOH – 10g of KOH in 100ml of distilled water

Procedure:-

Soft tissues was dissected and then were transferred into a clean petri dish and weighed. It was placed into a 100 ml conical flask and covered with aluminium foil. Microplastics extraction followed a modified alkali digestion method outlined by Dehaut et al., (2016) and Maes et al., (2017). In brief, this involved successive addition of 10% KOH (approximately 3x times the volume of the tissue) and small amount of 30% H_2O_2, incubating the mixture at 65°C for 24-48 hours for effective digestion of tissue to takes place. The cooled digestate was vacuum-filtered through Grade 1 cellulose membrane filter paper (pore size: 0.22 µm) and the filters were stored in separate covered petri dishes for further procedures.

3.5.3 Observation and validation of microplastics

Visual inspection was conducted on whole filters under a Carl Zeiss Stereo microscope (Stemi 508) using an imaging software (Micro Imaging GmbH), with images captured during the process and analysed by ZEN 3.9 (ZEN lite) software for further analysis. Microplastics were categorized into four morphotypes: fiber, pellet, film, and fragment, based on their physical attributes (Yan et al., 2021). The assessment included recording the number, size, shape, and color of microplastics in each sample.

Validation of the nature of microplastics was done by using Laser Micro Raman Spectrometer (Lab RAM HR Evolution, France) having spectral range 200 - 2100 nm and along with a microscope equipped with three objective; 5x, 10x, and 100x. Isopropanol was used as a clearing agent to avoid interferences of impurities on a filter paper. The measurements were carried out with a 532 nm (Nd-YAG laser 100 mW) excitation laser. To obtain the Raman images (spectra) and to perform the data analysis "Lab Spec 6 Horiba Scientific" software was used.

3.5.4 Coefficient of Microplastic Impact (CMPI) (Rangel-Buitrago et al., 2021).

CMPI quantifies the impact of specific microplastic shapes (e.g., fibers, pellets, fragments and films) by relating their abundance to the total microplastic concentration in a sample. The CMPI of each site was calculated using the following formula:

$$CMPI = \frac{\text{Specific MP Shape}}{\text{Total MPs}}$$

According to this methodology, microplastic impact can be classified ranging from minimal (0.0001–0.1), moderate (0.11–0.5), substantial (0.51–0.8), to severe (0.81–1) CMPI values.

3.6 GENOTOXICITY: COMET ASSAY

Single Cell Gel Electrophoresis (Wang *et al.*, 2009; Nagpure *et al.*, 2007)

Principle:

Cells in agarose gel undergo lysis to remove cellular proteins, leaving supercoiled DNA. Under alkaline/neutral conditions, the DNA unwinds. Electrophoresis separates broken DNA fragments from the main DNA, forming a comet-shaped structure. The extent of DNA migration from the head reflects DNA damage (D'Costa *et al.*, 2019).

Materials required:

Reagent bottles, beakers, conical flask, Whatmann filter paper, measuring cylinder, 1 ml syringe, coplin jars, microfuge tubes, double frosted slides, long coverslips, cotton, tissue, blotting paper, etc.

Chemical preparation:

1. **Phosphate buffer(0.01M):**

   Mix 0.8709 g of Dipotassium Hydrogen Phosphate and 0.6805 g of Potassium Hydrogen Phosphate in 400 ml Distilled Water. Adjust the pH to 7.2.make the volume to 500 ml.

2. **Phosphate buffer saline:**

   Weigh 4 g Sodium Chloride, 0.1 g Potassium Chloride, 0.72 g Sodium Phosphate Dibasic and 0.1225 g Potassium Phosphate Monobasic and mix in 400 ml Distilled water. Adjust the pH to 7.4. Make the volume to 500 ml with Distilled Water.

3. **Lysis buffer:**

   a) 10% Dimethylsulphoxide (DMSO): 10 ml DMSO added to 90 ml of Double Distilled Water

   b) 1% Triton X 100: Mix 0.1 ml triton x 100 in 9.9 ml Phosphate buffer saline(PBS)

   c) Stock solution: Weigh 14.602 g Sodium Chloride, 3.174 g EDTA Disodium Salt and 0.12 g Tris base and add to 70 ml Double Distilled Water. Stir the solution and add 0.8 g Sodium Hydroxide. Dissolve the mixture on a magnetic stirrer for 20 minutes. After all added compounds are completely dissolved, adjust the pH to 10. Then make the volume up to 89 ml with double distilled water. Store the solution at 5ºC.

   d) Working solution of Lysis Buffer:

   To prepare 100ml of lysis buffer :- Take 89ml of stock solution (c) to that add 10ml DMSO (a) and 1ml of Triton X 100 (b).

4. **Electrophopresis Buffer:**

   a) 10N NaOH: Add 20 g Sodium Hydroxide to 50 ml of Double Distilled Water

   b) 200 Mm EDTA: Mix 1.48 g of EDTA in 15 ml of Double Distilled Water. Adjust the pH to 10. Make the volume up to 20 ml and store at the room temperature.

   c) Working solution of Electrophoresis Buffer:

   Add 27 ml of solution, 4.5 ml of solution (b) and 1.8 ml Direct DMSO in 866.7 ml Double Distilled Water.

5. **Neutralizing Buffer:**

Weigh 4.845 g of Tris Base and add to 90 ml of Double Distilled Water. Adjust the pH to 7.5 and make volume upto 100 ml and store at room temperature but make it chilled before use.

6. **Preparation of Agarose Gel:**
   a) **High Melting Agarose:** 0.5 g High Melting Agarose is mixed in 50 ml Phosphate Buffer. Heat the solution in microwave oven until the Agarose powder is completely dissolved and the solution is clear. Keep it on a hot plate (LABQUEST BOROSIL hot plate) to avoid solidification of the mixture.
   b) **Low Melting Agarose:** 0.25 g of Low Melting Agarose is mixed with 50 ml Phosphate Buffer and heated till the agarose is dissolved.

7. **Ethidium Bromide Stain:** For 10x stock solution (20µg/ml) add 10 mg Ethidium Bromide in 50 ml Double Distilled Water. Stir the solution and store in amber bottle in dark. For 1x working solution add 1 ml stock solution in 9 ml Double Distilled Water.
8. **Anticoagulant solution:-**

   For preparing Citrate/EDTA buffer :-

   Weigh 0.45 M NaCI; 0.1 M glucose; 30 mM trisodium citrate; 26 mM citric acid and 10 mM EDTA and maintain the pH 4.6

Procedure:

For extraction of haemolymph :- Hemolymph (100 µL) was drawn from the ventral-sinus cavity of Littopenaeus vannamei using 1ml syringe pre-filled with 0.4 ml of citrate/EDTA buffer that had been cooled to 7°C, serving as an anticoagulant. It was then transferred into an eppendorf tube for using it for comet assay.

All the steps were performed under dim-light to prevent any photo-oxidation. First clean frosted glass slides were coated with a thin layer of High Melting Agarose gel and left to dry. Subsequently, a second layer comprising equal parts of sample and Low Melting Agarose was added and allowed to set briefly. Finally, a third layer consisting of Low Melting Agarose only

was applied, and the slides were left to dry thoroughly. Following preparation, the slides were immersed in a Lysis buffer for a 24-hour period and the slides were protected from light. After 24 hours, the slides were submerged into Electrophoresis for 20 min, then the electrophoresis unit was run for 20 min at 20 V/200 mA. Following electrophoresis, the slides were dried and subsequently immersed in a neutralizing buffer for 20 minutes. Then the slides were removed from the neutralizing buffer and excess liquid was removed using blotting paper. The slides were dried completely and stained with Ethidium Bromide. The coverslip was put on and then the slides were observed under fluorescence microscopy (OLYMPUS U-TV0.63×C).

3.7 BIOCHEMICAL ESTIMATIONS

3.7.1. PROTEIN ESTIMATION (Lowry *et al.*, 1951)

Principle:

In the presence of proteins, copper ions are reduced to cuprous ions, forming a blue-colored complex with the reagent under alkaline conditions. Additionally, tyrosine and tryptophan residues induce the reduction of phosphomolybdate and phosphotungstate, further enhancing sensitivity. Spectrophotometry measures the intensity of the blue color, directly correlating with protein concentration, typically at a specific wavelength (Shen, 2023).

Materials required:

Reagent bottles, beakers, conical flasks, micropipettes and tips, centrifuge tubes, test tubes, test tube stands, etc.

Chemical preparations:

1. **BSA (Bovine Albumin Serum) stock solution:**

   Mix 0.005 g of BSA in 20 ml 1N NaOH

2. **Lowry's Reagent:**

   a) **4% Sodium Bicarbonate (Na2Co3):**

      Weigh 4 g of Na2CO3 and mix with 100 ml Distilled water

   b) **4% Potassium Tartarate:**

      Add 0.2g Sodium Potassium Tartarate in 5 ml Distilled water.

   c) **2% CuSO4:**

      Add 0.1 g CuSO4 in 5 ml Distilled water.

Mix 96 ml of solution (a), 2ml of solution (b) and 2ml CuSO4 to prepare Lowry's reagent of 100ml.

3. **Folin's Reagent:**

   Mix 5 ml of Folin's reagent in 5ml Distilled water (1:1).

Procedure:

a) **Extraction:**

   Shrimp was dissected and the soft tissue was taken out (hepatopancreas and muscle). Soft tissues was weighed and homogenized with cold phosphate buffer saline and centrifuged at 4000rpm for 20 mins. The supernatant obtained is used for protein estimation.

b) **Estimation:**

   BSA stock solution was taken as a standard protein sample in 5 different concentrations for comparison with tissue samples. 1 ml of freshly prepared homogenate supernatant samples was taken. Both the standards and homogenate samples were treated with 5 ml Lowry's reagent and allowed to incubate at room temperature for 10 minutes. After the

incubation, 0.5 ml Folin's reagent was added to each test tube and incubated again at room temperature for 30 minutes. After the appearance of blue coloration the OD was measured at 660 nm using a spectrophotometer.

3.7.2. CATALASE ESTIMATION (Aebi, 1974)

Principle:

The catalase activity assay determines the rate at which the enzyme catalyzes the breakdown of H2O2 into H2O and O2. This is achieved by observing the reduction in absorbance of H2O2 at a particular wavelength. The rate of oxygen release during the catalase assay is directly assessed by monitoring the decline in H2O2 concentration spectrophotometrically at 610 nm, as the absorbance decreases with H2O2 decomposition (Beers and sizer1951).

Materials required:-

Reagent bottles, beakers, conical flasks, micropipettes and tips, test tubes, test tube stands, etc.

Chemical Preparation:-

1) Phosphate buffer(0.01M):

Mix 0.8709 g of Dipotassium Hydrogen Phosphate and 0.6805 g of Potassium Hydrogen Phosphate in 400 ml Distilled Water. Adjust the pH to 7.2.make the volume to 500 ml.

2) Catalase standard stock:

Dissolve 0.25 g Catalase Enzyme powder was dissolved in 5 ml Distilled Water

3) 0.2 M Hydrogen Peroxide (H2O2):

Mix 1.88 ml H2O2 in 98.12 ml Distilled Water

4) Dichromate Acetic Acid (1:3)

Add 10 ml Potassium Dichromate in 30 ml Glacial acetic acid. Mix the solution.

Procedure:-

Freshly prepared tissue homogenate was treated with 1ml Phosphate buffer, 0.5 ml 0.2 M H_2O_2 and 2 ml Dichromate Acetic Acid. After the addition, the samples were kept for incubation in a boiling water bath. Incubated samples were then used for catalase assay by measuring OD at 240 nm.

3.7.3. LIPID PEROXIDATION ASSAY - TBARS (Duarte-Restrepo *et al.*, 2020)

Principle:

The principle of the assay involves the reaction between lipid peroxidation byproducts, primarily malondialdehyde (MDA), and thiobarbituric acid (TBA). This reaction leads to the formation of MDA-TBA2 adducts, commonly known as Thiobarbituric Acid Reactive Substances (TBARS). The presence of TBARS yields a red-pink coloration, which can be quantified through spectrophotometric analysis at wavelength 535 nm. The concentration of TBARS is indicative of the oxidative damage to lipids and serves as a marker for oxidative stress in biological systems (Leon and Borges., 2020).

Materials required:

Reagent bottles, beakers, conical flasks, micropipettes and tips, test tubes, test tube stands, etc.

Chemical preparation :

1) Tris HCL Buffer:

Weigh 1.576 g Tris buffer and mix in 100 ml Distilled Water (pH 7 to 8).

2) Tichloroacetic acid (TCA)

Add 2.5g Trichloroacetic acid in 50ml Double distilled water.

3) Thiobarbituric acid (TBA)

Add 0.268g of Thiobarbituric acid powder was added to 40 ml of glacial acetic acid.

Procedure:

Freshly obtained tissues were homogenized in 5 ml TCA-HCl Buffer. Homogenate was added 1:1 (v:v) to trichloroacetic acid (TCA), and incubated on ice for 15 min. The solution was mixed at 2:1 ratio with TBA, and centrifuged at 2200 × g at 4˚C for 10 min. The whole supernatant was boiled for 10 min and refreshed at room temperature before the absorbance was recorded. The absorbance of clear supernatant was observed at wavelength 535 nm.

3.7.4. ALANINE TRANSFERASE (ALT) ESTIMATION (Reitman and Frankel, 1957)

Principle:

The ALT assay, or Alanine Transferase assay, diagnoses ALT enzyme levels in blood or tissue. It converts alanine to pyruvate with ALT, also converting α-ketoglutarate to glutamate. This yields equimolar NADH, measurable by spectrophotometry. Elevated ALT levels may signal tissue damage or illness (Hsueh *et al.*, 2011).

Materials required:

Reagent bottles, beakers, conical flasks, micropipettes and tips, test tubes, test tube stands, etc.

Chemical preparation:-

1) Standard Pyruvate:

0.0022g of Sodium pyruvate is dissolved in 10 ml Distilled Water

2) **ALT Substrate:**

Weigh 0.532 g of Alanine and 6 mg α-Ketoglutaric acid. Mix 0.1 ml of 1N NaOH in the weighed compounds and make the volume upto 20 ml using Phosphate Buffer.

3) **0.4 N Sodium Hydroxide (NaOH):**

Mix 3.2 g of NaOH in 200 ml of Distilled Water

4) **Di-Nitro-Phenyl Hydrazine (DNPH):**

Weigh 0.05 g of DNPH and mix with 46.4 ml Concentrated HCl. Adjust the volume to 20 ml using Distilled Water.

Procedure:

5 standard test tubes were prepared using different concentrations of standard pyruvate (0.2, 0.4, 0.6, 0.8, 1.0 ml) and the volume was made up to 1 ml using distilled water. Blank test tube with 1 ml distilled water was also prepared. For the experimental test tubes 0.1 ml from each sample was added. All the samples were treated with 0.5 ml ALT substrate in each and left for incubation at 37°C5 for 20 minutes. Then the samples were treated with 0.5 ml DNP Hydrazine and 5 ml of 0.4 N NaOH. After the additions the colour was noted and OD was taken at 540 nm.

3.7.5. ALKALINE PHOSPHATASE (ALP) ESTIMATION (King and Armstrong, 1934)

Principle:

The Alkaline Phosphatase Assay measures stress or toxin-induced damage. Elevated ALP activity reflects this stress response. The ALP assay evaluates Alkaline Phosphatase enzyme

activity by catalyzing the hydrolysis of a colorless substrate in the sample. This process leads to the production of a pale yellow-colored product (Adams *et al.*, 1997).

Materials required:

Reagent bottles, beakers, conical flasks, micropipettes and tips, test tubes, test tube stands, etc.

Chemical preparation:

1) **Glycine Buffer:**

   Add 0.375 g of Glycine and 0.035 ml HCl in 40 ml Distilled water. Adjust the pH to 3. Make the volume upto 50 ml using Distilled water.

2) **ALP Standards:**

   Mix 5 mg of p-nitrophenol in 5 ml of Distilled water

3) **0.025N Sodium Chloride (NaOH):**

   Mix 0.04 g of Sodium Hydroxide in 40 ml of Distilled water.

4) **ALP Standards**

   Weigh 0.375 g of Glycine, 0.166 g of NaOH, 0.01 g MgCl2 and 0.168 g of P-nitrophenol Phosphate and mix with 70 ml Distilled water. Adjust the pH to 9.2 and make the volume till 100 ml.

Procedure:

Standard samples were prepared at varying concentrations i.e., 0.1, 0.2, 0.3, 0.4, 0.5 ml and a glycine buffer was used to make up the volume. 1 ml of freshly prepared tissue homogenate was taken as a sample. Both standard and samples were treated with 0.2 ml ALP substrate and were allowed to incubate at 37°C for 15 minutes. After incubation, 5 ml of 0.025 N NaOH was added in the sample mixtures and the OD was measured at 405nm.

3.8 CONDITION INDEX (Sharawy et al., 2017)

Principle:-

The condition factor or index assesses the overall health of crustaceans, assuming that heavier individuals of the same length are in healthier condition than less weightier individuals. It reflects the relationship between weight and length, indicating the shrimp's health and growth status.

Materials required:-

Measuring scale and weighing balance

Estimation:-

Shrimp with intact rostrums were manually straightened, and a thread was aligned dorsally from the rostrum tip to the telson end for total length measurement (L). The wet weight (W) was determined using an electronic digital balance. Condition Index was calculated using the formula for total length – weight relationship as follow :-

$$K=\frac{Wt}{TL^3}\times 100$$

Where,

K= Condition factor

Wt= Wet body weight in g

TL= Total length in cm

3.9 STATISTICAL ANALYSIS

All the statistical analysis was done using Graphpad Prism software. A significance level of 0.05 was chosen. The normality of the data was assessed using the Shapiro-Wilk Normality test. A two-way ANOVA, along with a Tukey's Multiple Comparison Test for post-hoc analysis, was conducted to analyze various biochemical and physiological factors. Graphs was drawn to interpret the microplastic data by using Microsoft Excel. Pearson’s Coorelation Matrix was performed for biochemical and physiological tests (Catalase assay, Protein of Hepatopancreas, ALT Test, Protein of Muscles, ALP test, TBARS Assay and Condition Index) for a each study site across the three month.

PLATE –1

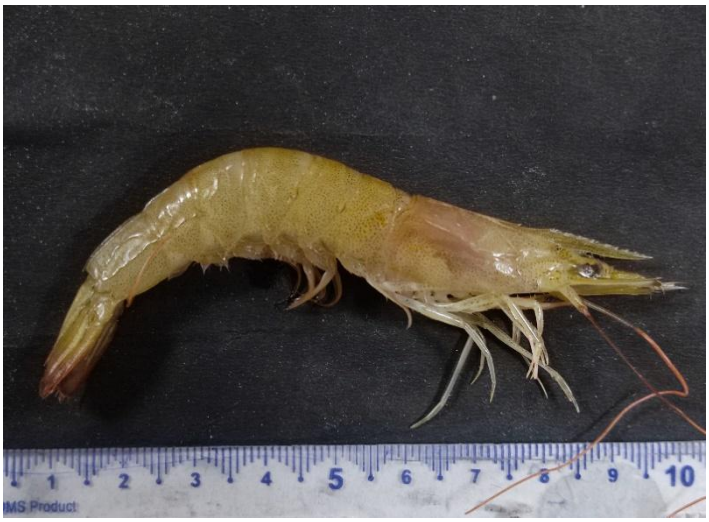


Fig 1: Study animal – Whiteleg Shrimp (*Litopenaeus vannamei*)

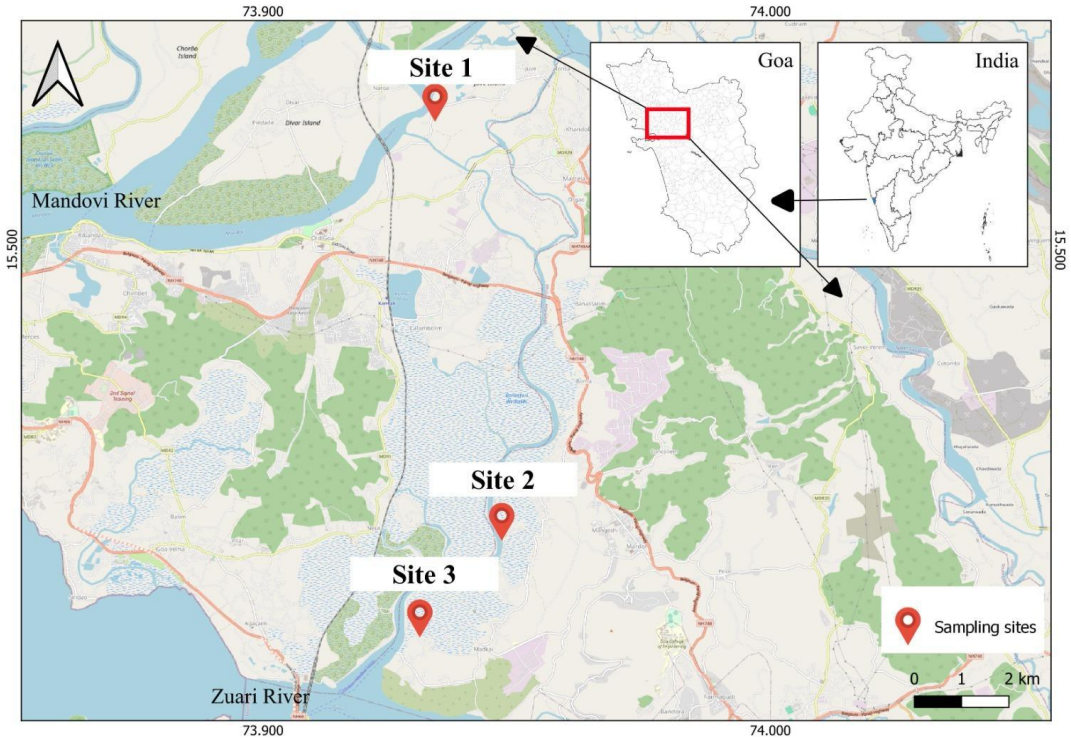


Fig 2: Map of the study area:

Site 1 - Saint Estevam Island, Site 2 - Cundaim and Site 3 – Madkaim

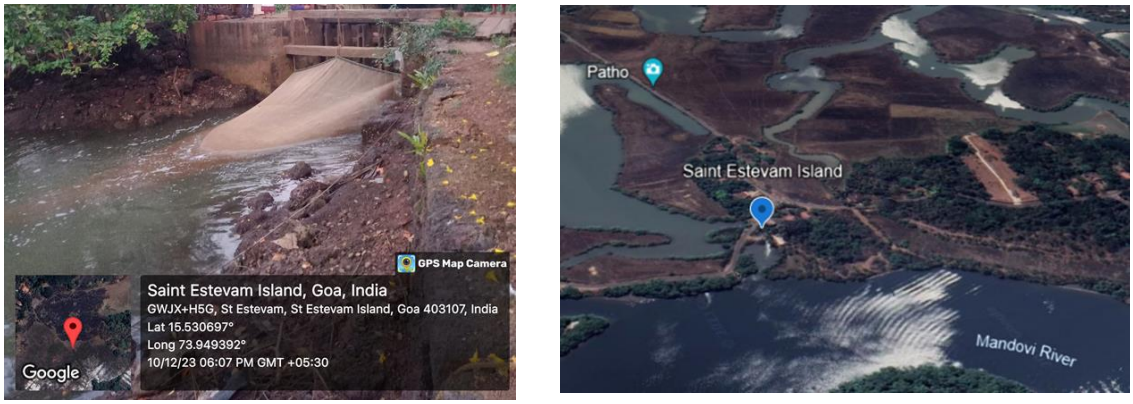


Fig 3: Images of Sluice gate and study site 1 - Saint Estevam Island

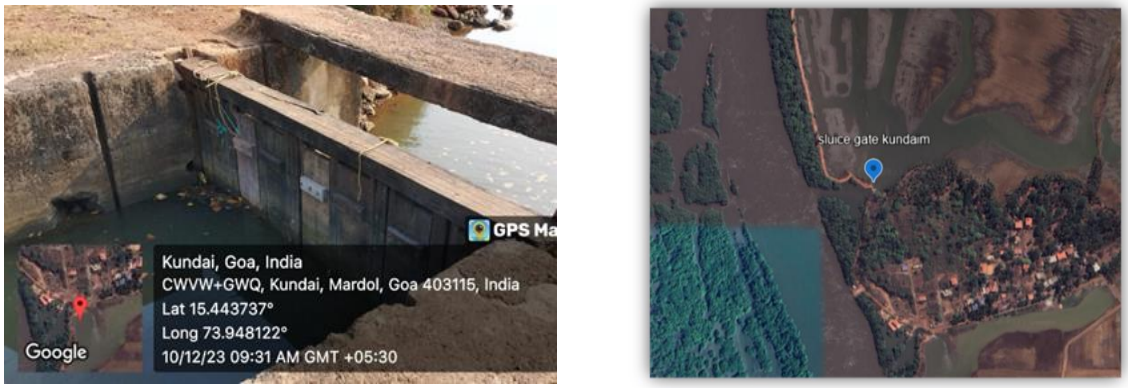


Fig 4: Images of Sluice gate and study site 2 – Cundaim

Fig 5: Images of Sluice gate and study site 3 - Madkaim

CHAPTER 4

ANALYSIS AND CONCLUSION

4.1 RESULTS

4.1.1 Abundance of microplastic in tissue and water sample

Accumulation of microplastics were observed and detected in hepatopancreas of shrimps and water samples from sluice gate harvest sites across three different sites: Saint Estevam Island, Cundaim and Madkaim over a three months depicted in fig 1 and fig 2 showing the abundance of microplastic across the three different sites. Saint Estevam Island exhibits a higher concentration of microplastics in comparison to the adjacent sites of Cundaim and Madkaim. The presence and accumulations of microplastic varies over a period of three months ie. January, February and March across the sites.

4.1.2 Shapes of microplastic

Various forms of microplastics, including fragments, fibers, films, and pellets, were observed under the stereomicroscope. These microplastic shapes were identified in both hepatopancreas of shrimp and water samples collected from three distinct sites: Saint Estevam Island, Cundaim, and Madkaim, over a period of three months. This observation is detailed in the fig 3 & 4, fig 5 & 6 and fig 7 & 8 for shapes of microplastic in hepatopancreas and water samples respectively. Fragments were identified as the prevailing shape of microplastics in shrimp hepatopancreas samples, followed by fibers, pellets, and film. Conversely, fibers were predominantly observed in water samples, with other shapes present to a lesser extent, at Saint Estevam Island. Fragments were observed as dominant shape followed by fibers, pellets and then film in tissue as well as water samples at Cundaim. Fibers were identified as the predominant form of microplastics, followed by fragments, pellets, and films at Madkaim.

4.1.3 Sizes of microplastics

Different sizes ranges were observed and measured by using Zeiss software ranging from 1 to 1500µm. Fig 9 and 10 depicts the sizes of microplastic in hepatopancreas and water samples across three months at three different sites. Specifically, microplastics within the 1-50µm range predominated in abundance, while those falling within the 100-1500µm range were comparatively less.

4.1.4 Colour of microplastics

Diverse colour were observed of microplastics such as Transparent, White, Blue, Yellow, Pink and Red. In Fig 11, pie chart depicting the percentage of microplastic colours in hepatopancreas of shrimp as well as water samples at Saint Estevam Island, Cundaim and Madkaim. This observation is detailed in the fig 11 & 12, fig 13 & 14 and fig 15 & 16 for colours of microplastic in hepatopancreas and water samples respectively at three distinct sites. Across all sites, blue was the dominant color, displaying a higher percentage, followed by transparent and white colours. Pink, red, and yellow colors were observed in smaller proportions.

4.1.5 Polymer Types

The nature of various polymers were analysed and validated by Micro-Raman Spectrophotometer. Polymers found in the hepatopancreas of shrimps as well as water samples across three sites were Polyethylene Terephthalate (PET), Polyamide, Polycarbonate, Poly Vinyl Chloride (PVC), Polyacrylonitrile, Polyethylene Vinyl Acetate, Epoxy, Polyethelene, and Thermoplastic polyester. Polymers were confirmed by comparing the unknown sample with standard spectra.

4.1.6 Coefficient of Microplastic Impact (CMPI)

Figure 17 and 18 illustrate the Coefficient of Microplastic Impact (CMPI) values ranging from 0.0001 to 1, derived by categorizing microplastic shapes into minimum, moderate, substantial, and severe impacts. This classification is based on the cumulative presence of microplastics across three months at distinct sites in hepatopancreas of shrimps. In fig 17, at Saint Estevam Island and Cundaim, the impact of fibers, pellets, and fragments was categorized as moderate, while films exhibited a minimum impact. Conversely, at Madkaim, fibers demonstrated a substantial impact, fragments were classified as moderate, and pellets and films showed a minimum impact.

Figure 18, shows the impact assessment of fibers, pellets, films and fragments in water samples collected from Saint Estevam Island, Cundaim, and Madkaim. Notably, at Saint Estevam Island and Cundaim, the impact of fibers, pellets, and fragments was categorized as moderate. At Madkaim, samples revealed a substantial impact specifically from fibers, whereas fragments and pellets demonstrated a moderate impact. Meanwhile, across all sites, the impact of films remained minimal.

4.1.7 Genotoxicity - Comet Assay

To validate our hypothesis on shrimp genotoxicity, comet assays were performed at three sites: Saint Estevam Island, Cundaim, and Madkaim. The findings of this study indicate the absence of DNA damage, as evidenced by the absence of comet formations in the comet assays, with the exclusive detection of intact hemocytes.

4.1.8 Biochemical Estimation

For all the biochemical tests two – way ANOVA was used to check the diffrences between three different sites across the months.

1. The analysis revealed significant differences in protein concentration in muscle tissue across three months at three different sites. Interaction between months and sites significantly contributed to the overall variation in protein levels ($F = 13.23$, $p < 0.0001$), indicating that the effect of months on protein estimation varied depending on the site. Additionally, protein levels varied significantly across months ($F = 12.10$, $p = 0.0003$), with some months showing consistently higher or lower levels. However, the effect of sites alone on protein estimation was not significant ($F = 2.686$, $p = 0.1007$). Tukey's multiple comparisons test was conducted to examine differences in protein estimation and are depicted in the fig 19.

The two-way ANOVA analysis on hepatopancreas protein levels in shrimp reveals significant effects for both the interaction between Months and Sites ($F = 16.85$, $p < 0.0001$) and for the main effects of Months ($F = 44.23$, $p < 0.0001$) and Sites ($F = 24.47$, $p < 0.0001$). This indicates that both the Months and the different sites have a substantial impact on the protein levels observed. Fig 20 displays the results of Tukey's multiple comparisons test, llustrating the distinctions in protein estimation among various groups.

2. The analysis conducted on catalase enzyme estimation reveals statistically significant findings across various factors. The results demonstrate a significant interaction effect between months and sites ($F = 20.56$, $p < 0.0001$), indicating that the combined influence of these two factors

contributes to the total variation observed. Additionally, both months ($F = 5.634$, $p = 0.0136$) and sites ($F = 6.883$, $p = 0.0076$) individually exhibit significant difference. The Tukey's multiple comparisons test was utilized to explore differences in catalase enzyme levels across three distinct sites over three consecutive months depicted in the fig 21.

3. The influence of months and sites on lipid peroxidation levels assessed through the TBARS assay. The analysis reveals the interaction between months and sites is insignificant ($F = 2.648$, $p = 0.0527$). The ANOVA test revealed significant effects of both months ($F = 24.75$, $p < 0.0001$) and sites ($F = 56.38$, $p < 0.0001$) on the outcome variable, indicating that the average outcome varies statistically across different months and sites. The Tukey's multiple comparisons test was done to show the variations across sites with months and is depicted in the fig 22.

4. The analysis of Alanine Transferase (ALT) estimation, a marker of hepatotoxicity in shrimps, across different sites revealed significant variations. Both the interaction between months and sites ($F= 8.883$, $p < 0.0001$) and the main effects of months ($F = 69.90$, $p < 0.0001$) and sites ($F = 23.59$, $p < 0.0001$) were highly significant. The Tukey's multiple comparisons test highlighted significant variance in alanine transferase (ALT) estimation between sites across the three months in the fig 23.

5. The analysis of Alkaline Phosphatase (ALP) in shrimps across different sites over three months revealed significant variations. Both the interaction between months and sites ($F= 15.99$, $p < 0.0001$) and the main effects of months ($F = 8.056$, $p = 0.0089$) and sites ($F = 7.042$, $p = 0.0070$) were statistically significant. Results from Figure 24 show statistically significant differences determined by Tukey's multiple comparisons test in the levels of alkaline phosphatase (ALP) measured across various sites over the three-month study period.

4.1.9 Condition Index

The results of the two-way repeated measures ANOVA indicate that there is a significant interaction effect between months and sites ($F = 4.416$, $p = 0.0020$). However, the main effect of months alone is not significant ($F = 0.2694$, $p = 0.6996$). Similarly, the main effect of sites alone is not significant ($F = 0.2518$, $p = 0.7780$), suggesting that the differences between sites. The Tukey's multiple comparisons test was utilized to explore differences in condition index of shrimps across three distinct sites over three consecutive months depicted in the fig 25.

4.1.10 Pearson's Coorelation Test

The correlation was analysed for seven different tests (Catalase assay, Protein of Hepatopancreas, ALT Test, Protein of Muscles, ALP test, TBARS Assay and Condition Index) for a each study site across the three months.

According to Pearson's correlation test at Saint Estevam Island showed in Table 1, Notably, there is a significant negative correlation between CAT activity and ALT levels ($p < 0.01$**, $r = -0.611$). Similarly, CAT activity exhibits a significantly negative correlation with TBARS levels ($p < 0.01$**, $r = -0.621$). ALT levels show a significantly positive correlation with TBARS assay results ($p < 0.001$***, $r = 0.999$).

At Cundaim, the Pearson's correlation test, as depicted in Table 2. There is a positive correlation between CAT activity and ALP levels ($p < 0.01$**, $r = 0.554$). Similarly, CAT activity displays a significant positive correlation with TBARS levels ($p < 0.001$***, $r = 0.615$). Additionally, Protein (M) exhibits a significant negative correlation with ALT ($p < 0.01$**, $r = -0.727$). ALT activity demonstrates a significantly positive correlation with ALP levels ($p < 0.01$**, $r = 0.550$). ALT

levels also exhibit a significantly positive correlation with TBARS assay results ($p < 0.001$***, $r = 0.604$). Additionally, ALP shows a positive correlation with TBARS ($p < 0.01$**, $r = 0.558$).

Table 3 represents Pearson's correlations at Madkaim. CAT exhibits negative correlations with protein (H) ($p < 0.01$**, $r = -0.590$), ALT ($p < 0.05$*, $r = -0.530$), and TBARS Assay ($p < 0.05$*, $r = -0.517$). However, CAT shows a positive correlation with ALP ($p < 0.01$**, $r = 0.548$). Protein (H) exhibits a positive correlation with Protein (M) ($p < 0.01$**, $r = 0.660$) and ALP levels ($p < 0.001$***, $r = 0.801$). Protein (M) also shows a positive correlation with ALP ($p < 0.001$***, $r = 0.792$). Additionally, ALP shows a positive correlation with TBARS ($p < 0.05$*, $r = 0.476$).

GRAPHS

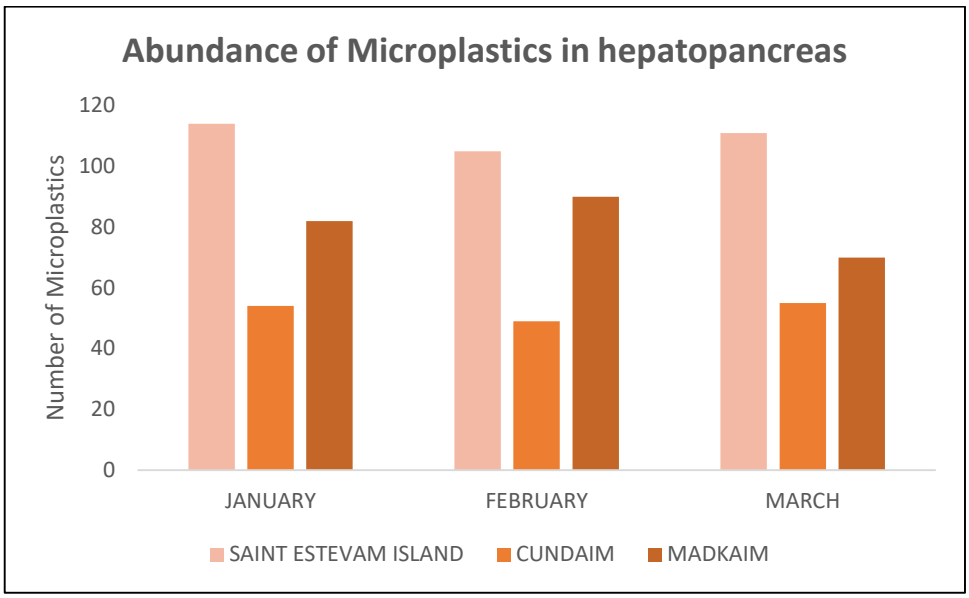


Fig 1 :- Abundance of microplastics found in hepatopancreas of shrimps collected from three different sites over a three months

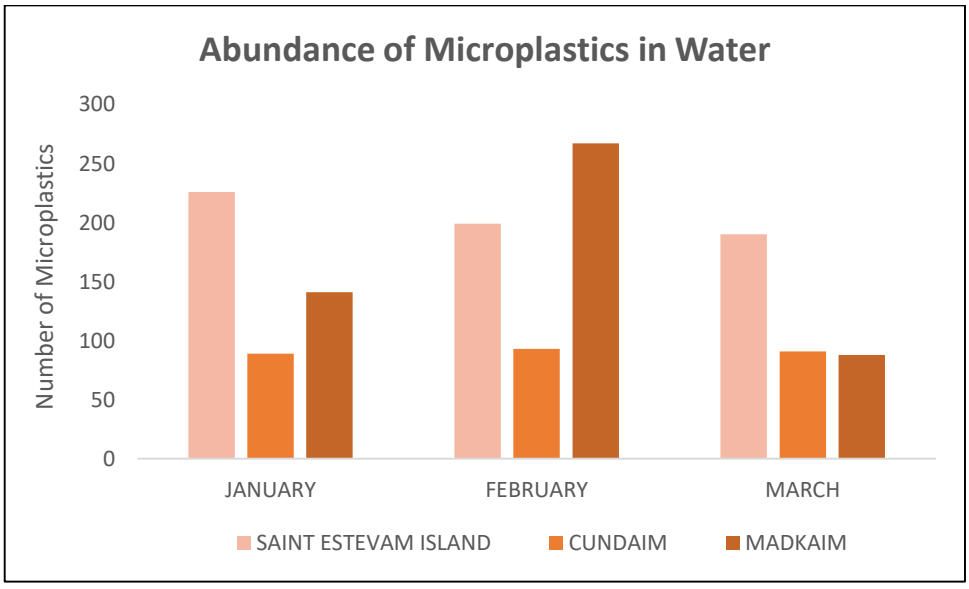
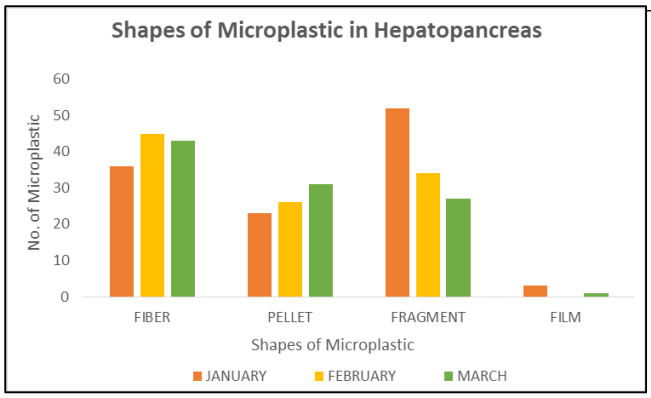


Fig 2 :- Abundance of microplastics found in water samples collected from three different sites over a three months



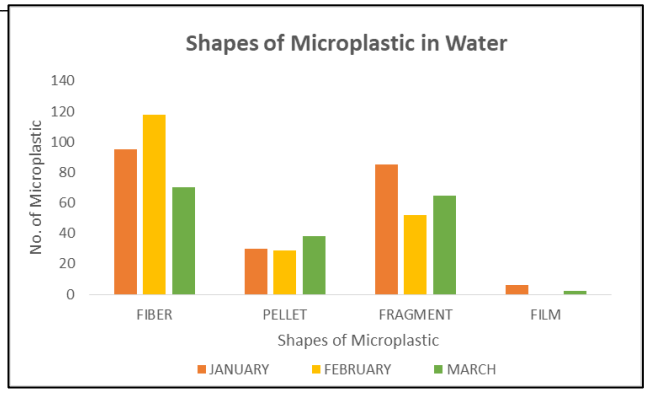
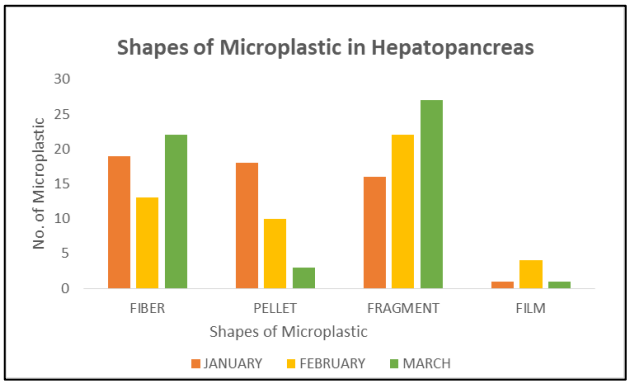


Fig 3 & 4 :- Shapes of Microplastic in hepatopancreas of shrimp and water sample at Saint Estevam Island



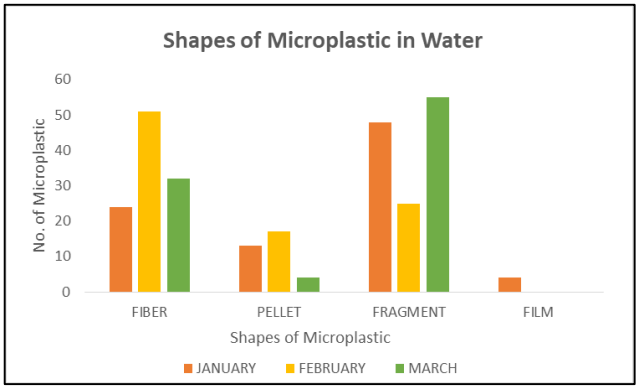
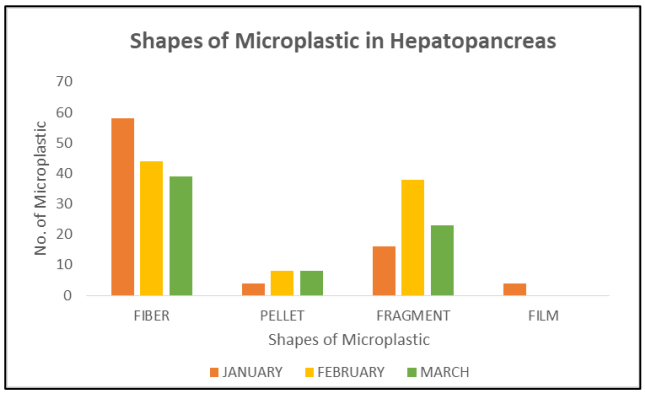


Fig 5 & 6:- Shapes of Microplastic in hepatopancreas of shrimp and water sample at Cundaim



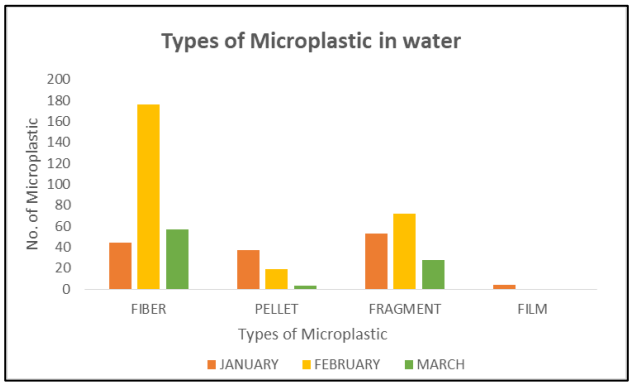


Fig 7 & 8:- Shapes of Microplastic in hepatopancreas of shrimp and water sample at Madkaim

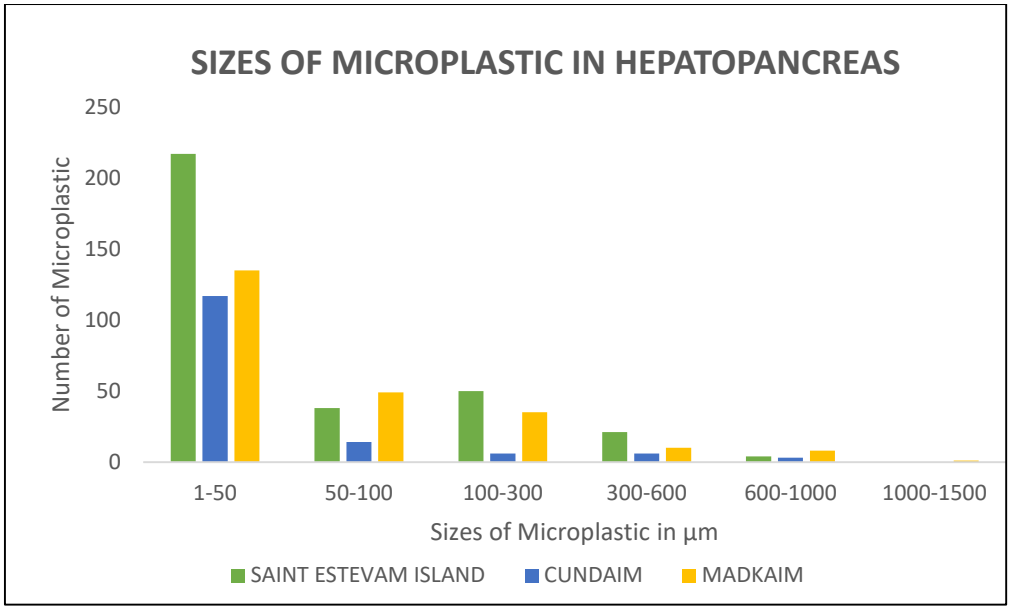


Fig 9:- Microplastics of diverse sizes were measured within the hepatopancreas at three different sites over a period of three months.

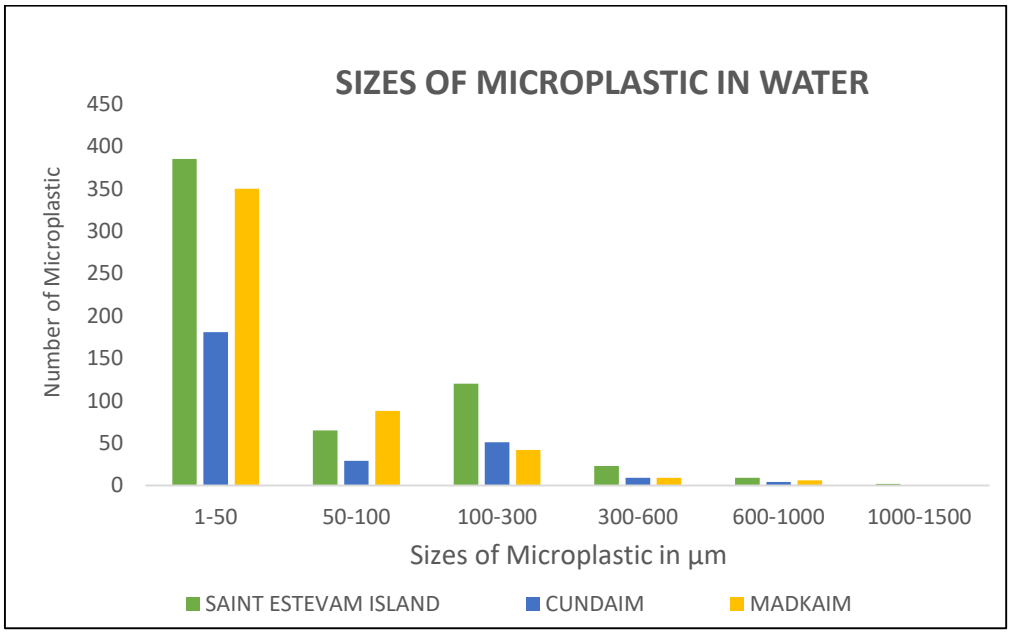
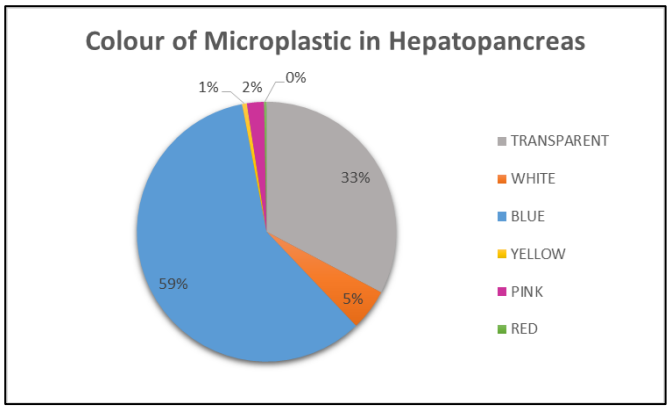


Fig 10 :- Microplastics of diverse sizes were measured in water samples collected from three distinct sites over a period of three months



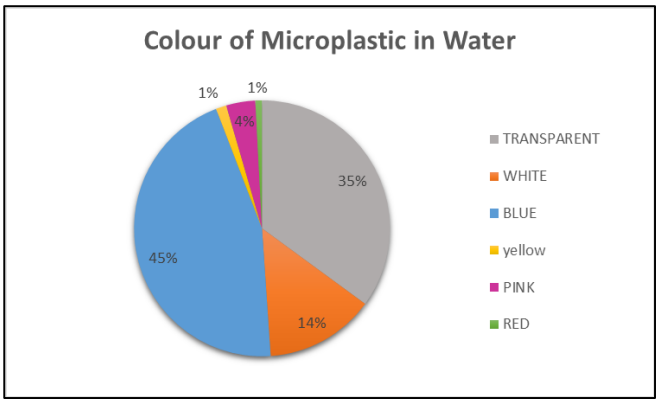
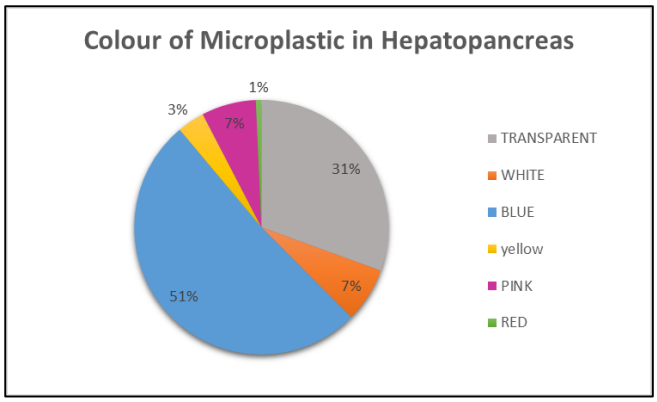


Fig 11 & 12 :- Pie chart depicting the percentage of microplastic colours found in hepatopancreas of shrimp and water sample at Saint Estevam Island



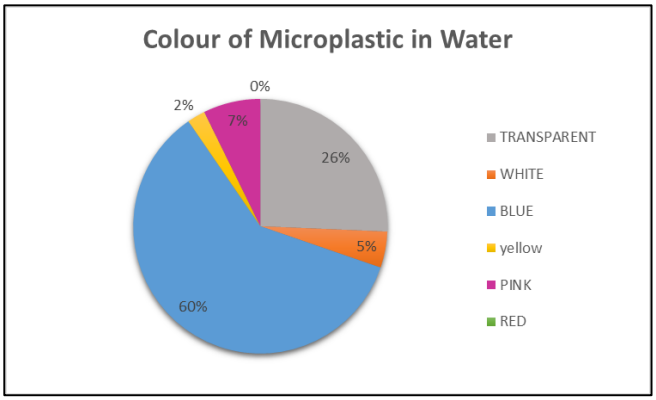


Fig 13 & 14:- Pie chart depicting the percentage of microplastic colours found in hepatopancreas of shrimp and water sample at Cundaim

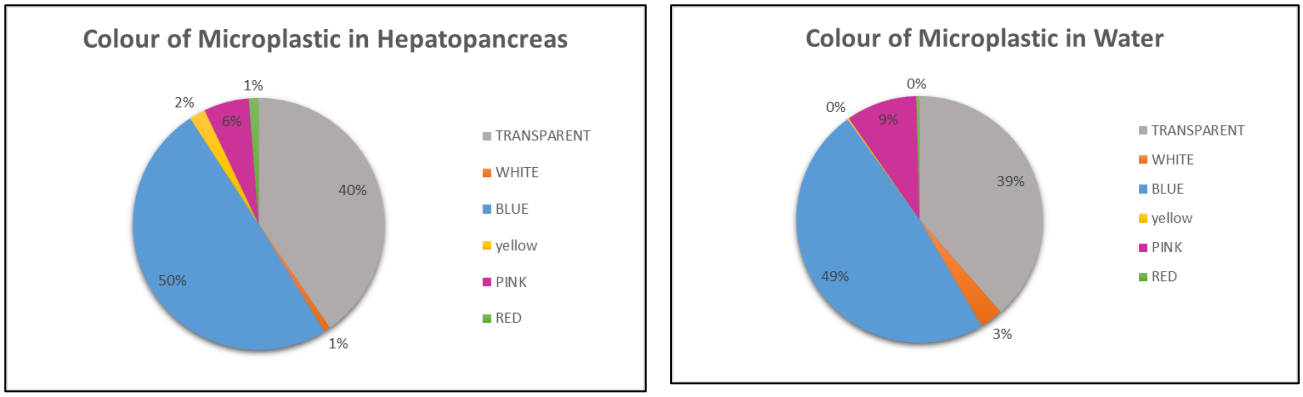


Fig 15 & 16 :- Pie chart depicting the percentage of microplastic colours found in hepatopancreas of shrimp and water sample at Madkaim

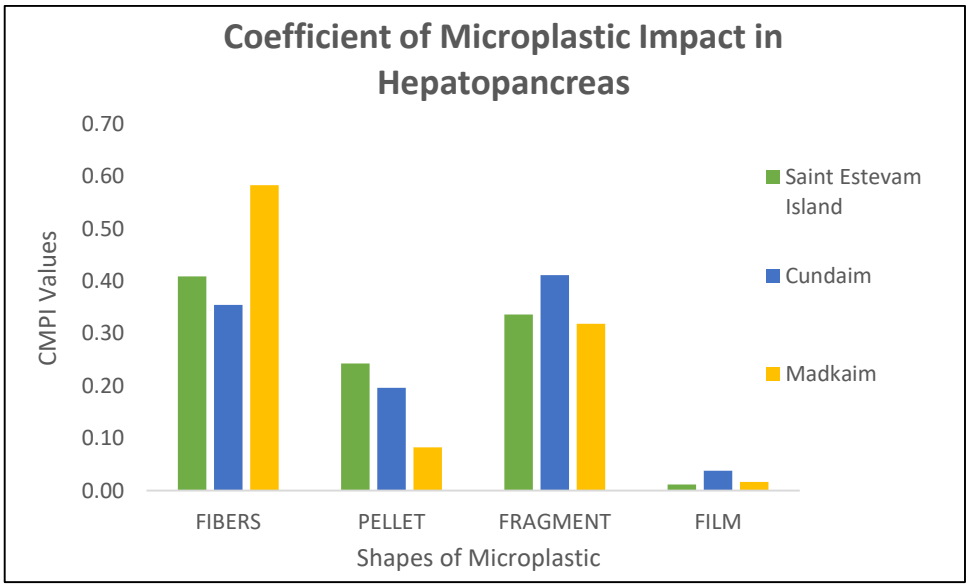


Fig 17:- Coefficient of Microplastic Impact (CMPI) in hepatopancreas of shrimps across three sites over a period of three months

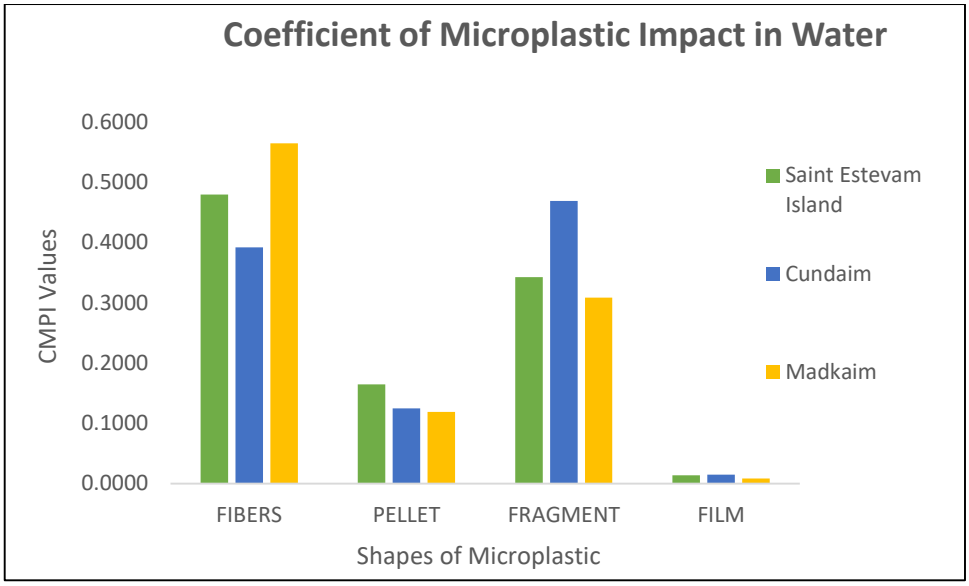


Fig 18:- Coefficient of Microplastic Impact (CMPI) in water samples across three sites over a period of three months

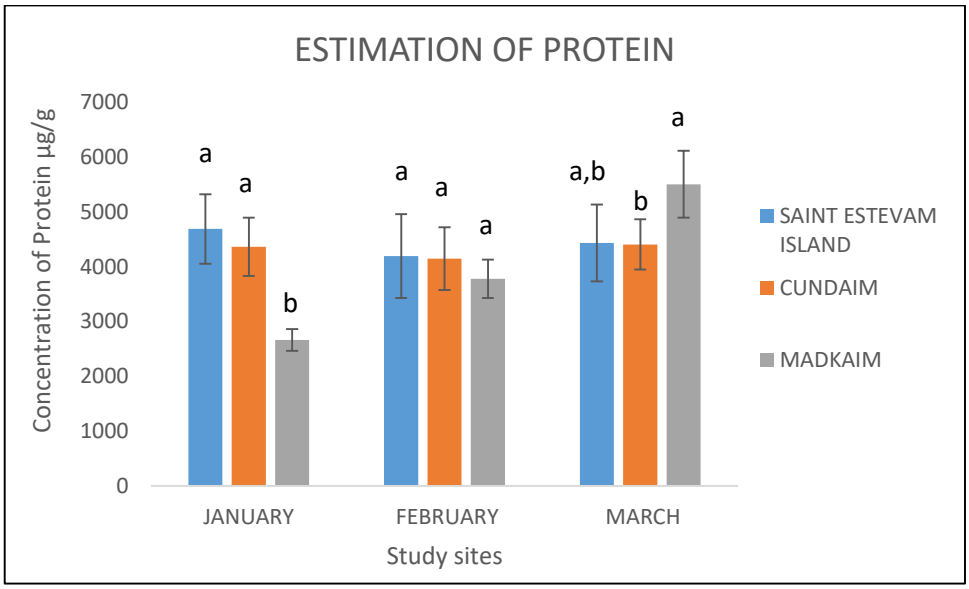


Fig 19:- Estimation of protein in the muscle of shrimps collected from the sampling sites. Two way ANOVA with Tukey test ($F = 13.23$, $p < 0.0001$). The data are expressed as the mean ± STD. Notes: Different letters indicate significant differences ($p < 0.05$).

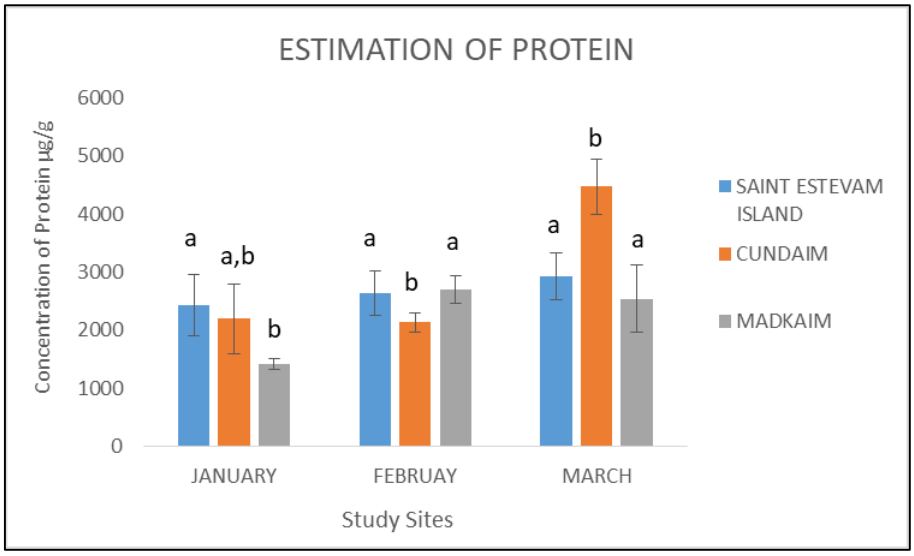


Fig 20:- Estimation of protein in the hepatopancreas of shrimps collected from the sampling sites. Two way ANOVA with Tukey test for ($F = 16.85$, $p < 0.0001$). The data are expressed as the mean ± STD. Notes: Different letters indicate significant differences ($p < 0.05$)

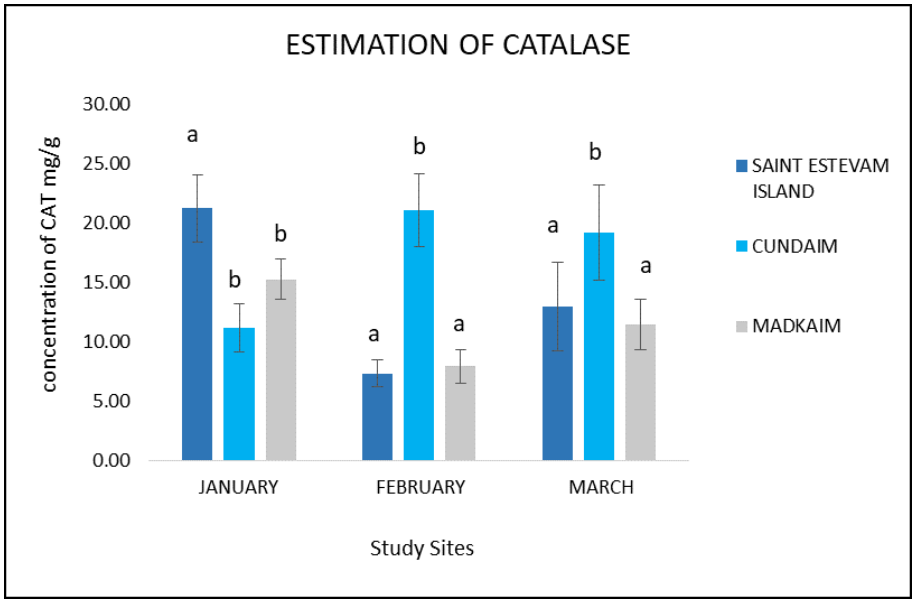


Fig 21:- Catalase estimation in shrimps collected from the sampling sites . Two way ANOVA with Tukey test for Catalase enzyme activity ($F = 20.56$, $p < 0.0001$), in the hepatopancreas of L. vannamei. The data are expressed as the mean ± STD. Notes: Different letters indicate significant differences ($p < 0.05$)

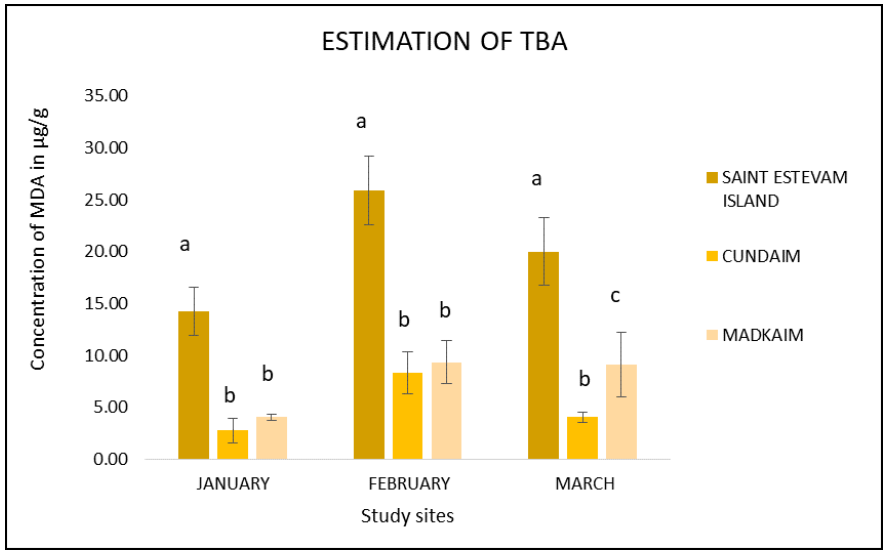


Fig 22:- Lipid Peroxidation estimation in shrimps collected from the sampling sites. Two way ANOVA with Tukey test for lipid peroxidation (F= 2.648, p = 0.0527) in the hepatopancreas. The data are expressed as the mean ± STD. Notes: Different letters indicate significant differences ($p < 0.05$)

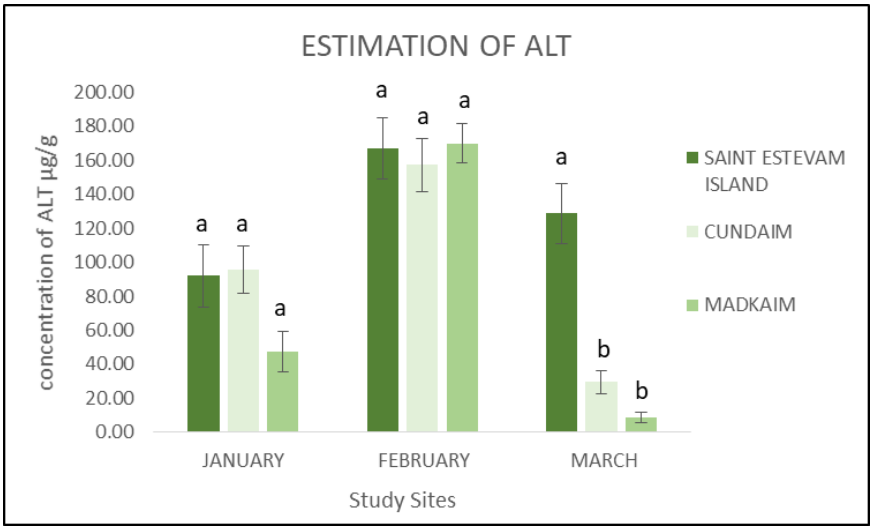


Fig 23:- Estimation of Alanine Transferase in shrimps collected from the sampling sites. Two way ANOVA with Tukey test (F = 8.883, p < 0.0001) in the hepatopancreas. The data are expressed as the mean ± STD. Notes: Different letters indicate significant differences ($p < 0.05$)

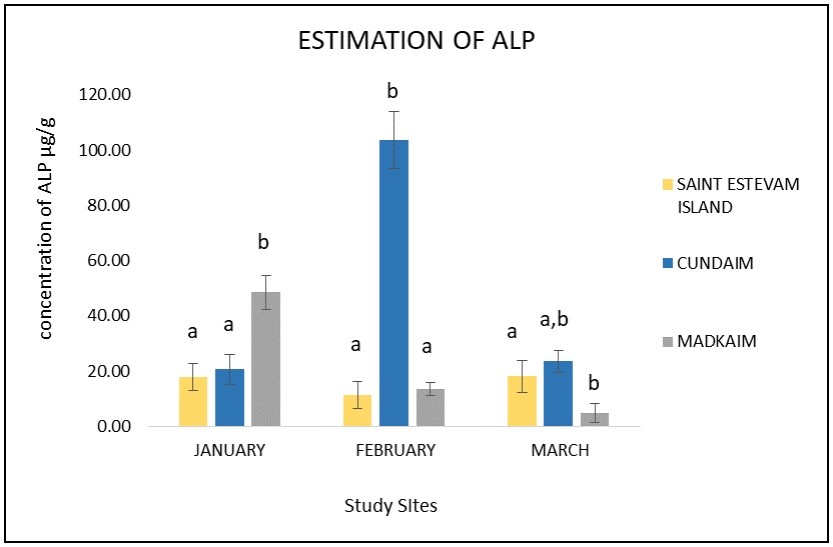


Fig 24:- Estimation of Alkaline Phosphatase in shrimps collected from the sampling sites. Two way ANOVA with Tukey test ($F = 15.99$, $p < 0.0001$) in the hepatopancreas. The data are expressed as the mean ± STD. Notes: Different letters indicate significant differences ($p < 0.05$)

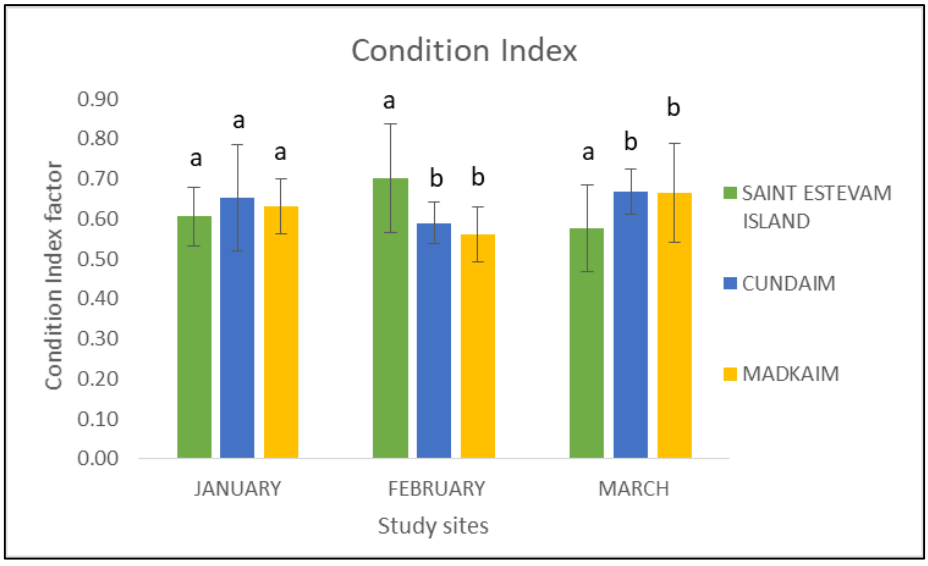


Fig 25:- Condition index of shrimps collected from the sampling sites. Two way ANOVA with Tukey test for condition index ($F = 4.416$, $p = 0.0020$). The data are expressed as the mean ± STD. Notes: Different letters indicate significant differences ($p < 0.05$)

TABLES

	CAT	PROTEIN (H)	ALT	PROTEIN (M)	ALP	TBARS	CONDITION INDEX
CAT	-						
PROTEIN (H)	-0.137	-					
ALT	-0.611**	-0.054	-				
PROTEIN (M)	0.332	0.074	-0.245	-			
ALP	0.309	-0.225	-0.077	0.038	-		
TBARS	-0.611**	-0.054	0.999***	-0.245	-0.077	-	
CONDITION INDEX	0.310	-0.108	0.024	0.177	0.047	0.024	-

Table 1 : Pearson's Correlation of *L. vannamei* collected from Saint Estevam Island over a three months

	CAT	PROTEIN (H)	ALT	PROTEIN (M)	ALP	TBARS	CONDITION INDEX
CAT	-						
PROTEIN (H)	0.087	-					
ALT	0.171	-0.727***	-				
PROTEIN (M)	-0.167	0.225	-0.286	-			
ALP	0.554**	-0.360	0.550**	-0.462	-		
TBARS	0.615***	-0.223	0.604***	-0.196	0.558**	-	
CONDITION INDEX	0.183	0.027	-0.305	0.307	-0.138	-0.098	-

Table 2 : Pearson's Correlation of *L. vannamei* collected from Cundaim over a three months

	CAT	PROTEIN (H)	ALT	PROTEIN (M)	ALP	TBARS	CONDITION INDEX
CAT	-						
PROTEIN (H)	-0.590**	-					
ALT	-0.530*	0.350	-				
PROTEIN (M)	-0.244	0.660**	-0.324	-			
ALP	0.548**	-0.801***	-0.091	-0.792***	-		
TBA	-0.517*	0.433	0.202	0.330	0.476*	-	
CONDITION INDEX	7.0E-05	-0.198	0.110	-0.339	0.329	-0.178	-

Table 3 : Pearson's Correlation of *L. vannamei* collected from Madkaim over a three months

PLATE -2

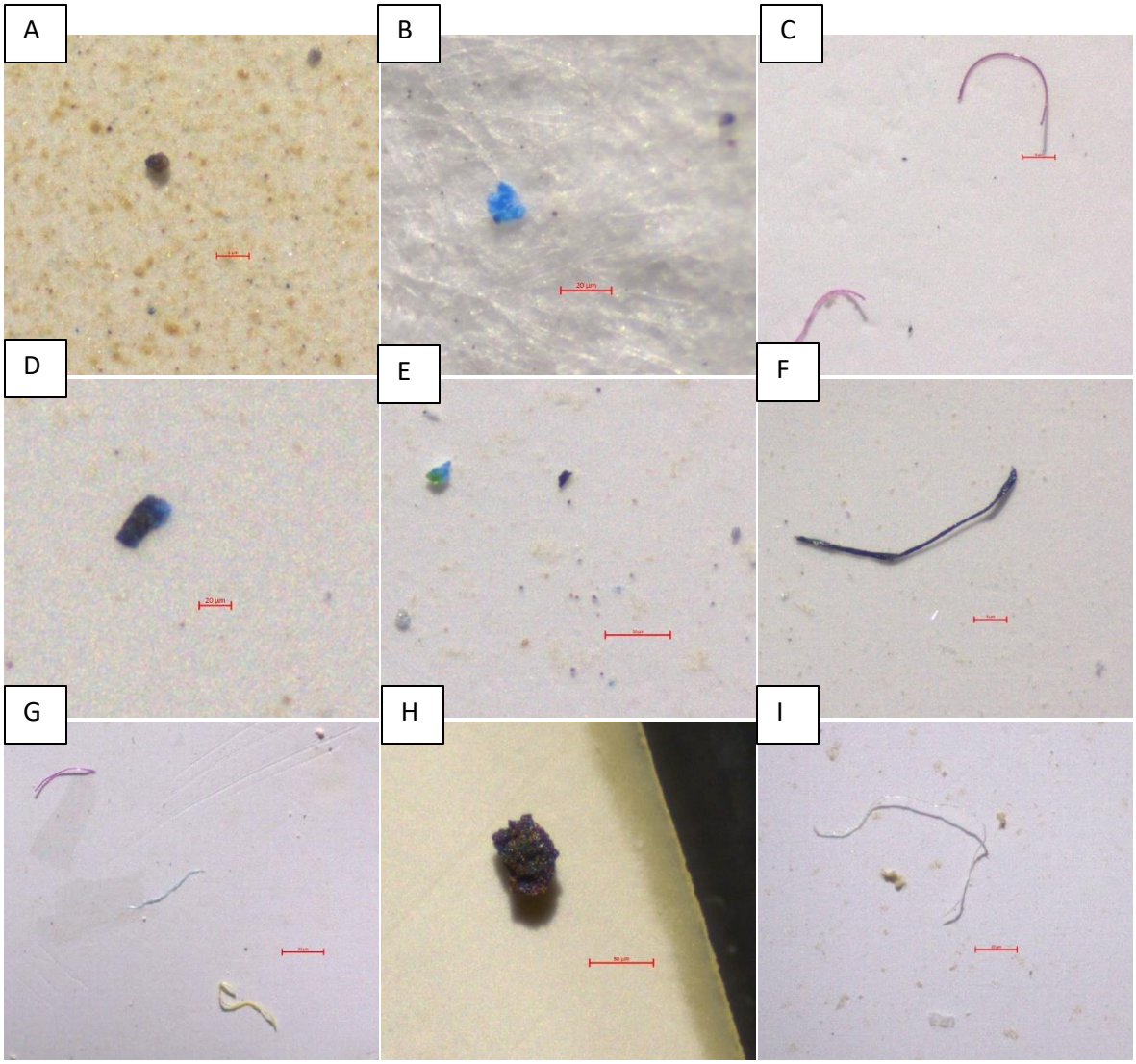


Fig 1: Microplastic detected in hepatopancreas of shrimp and water at study site

(A-Pellet; B,E,D- Fragment; D- Film; C,F,G,I- Fiber)

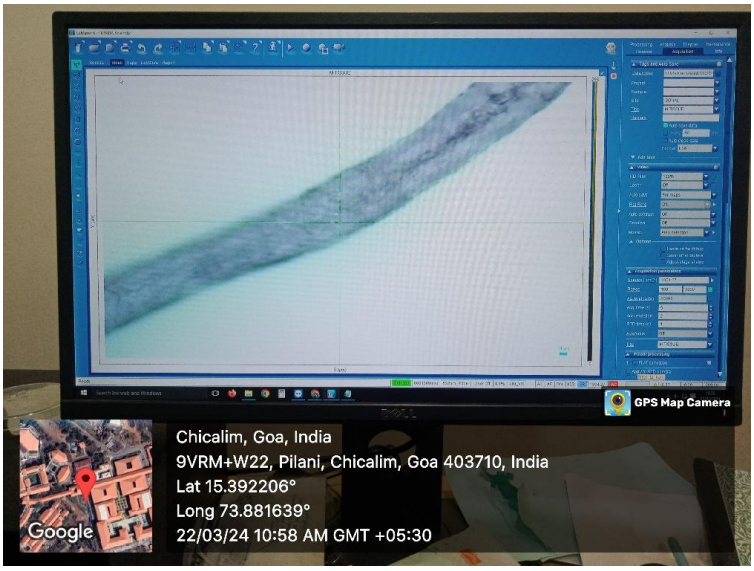


Fig 2 :- Validation of microplastic by using μ-Raman spectrophotometer using Lab 6 Spec Horiba scientific software

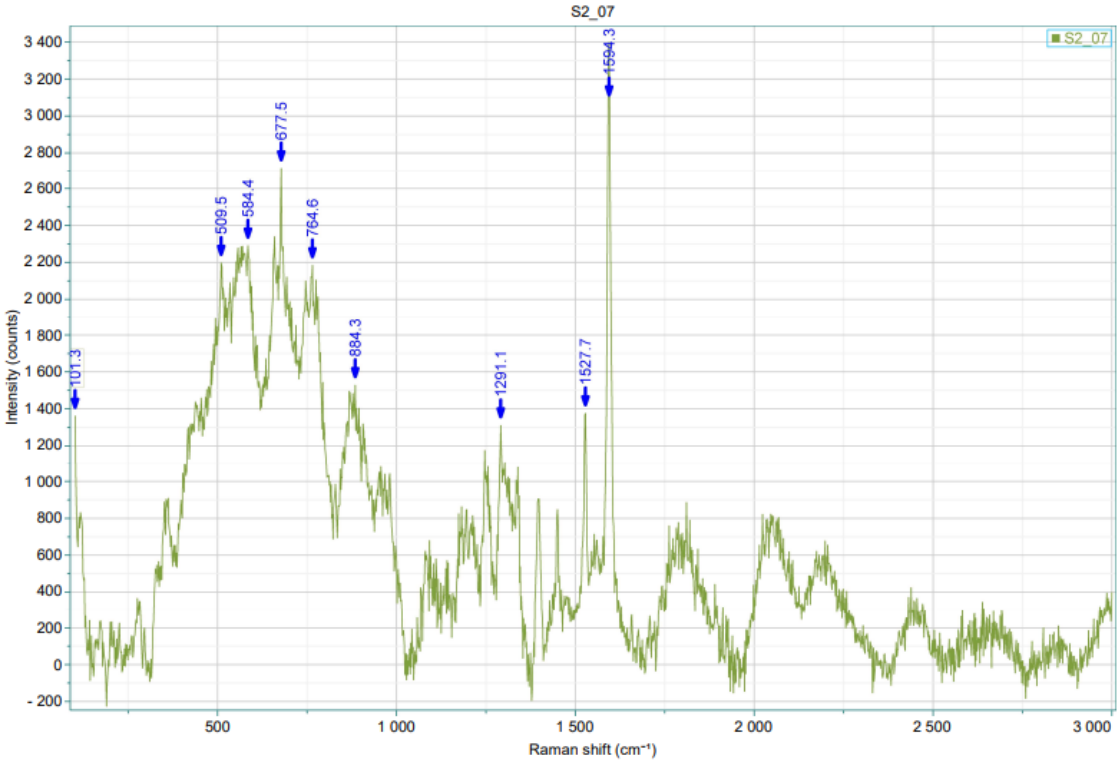


Fig 3 :- Raman Spectra of unknown microplastic identified as a Polycarbonate

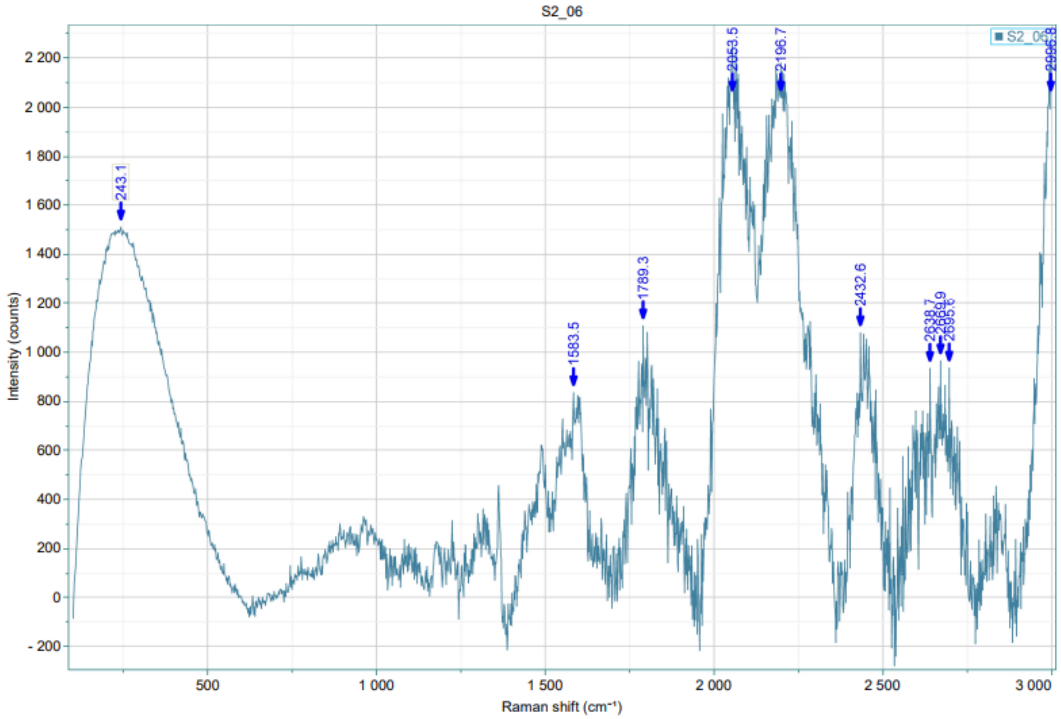


Fig 4 :- Raman Spectra of unknown microplastic identified as a Polyacrylonitrile

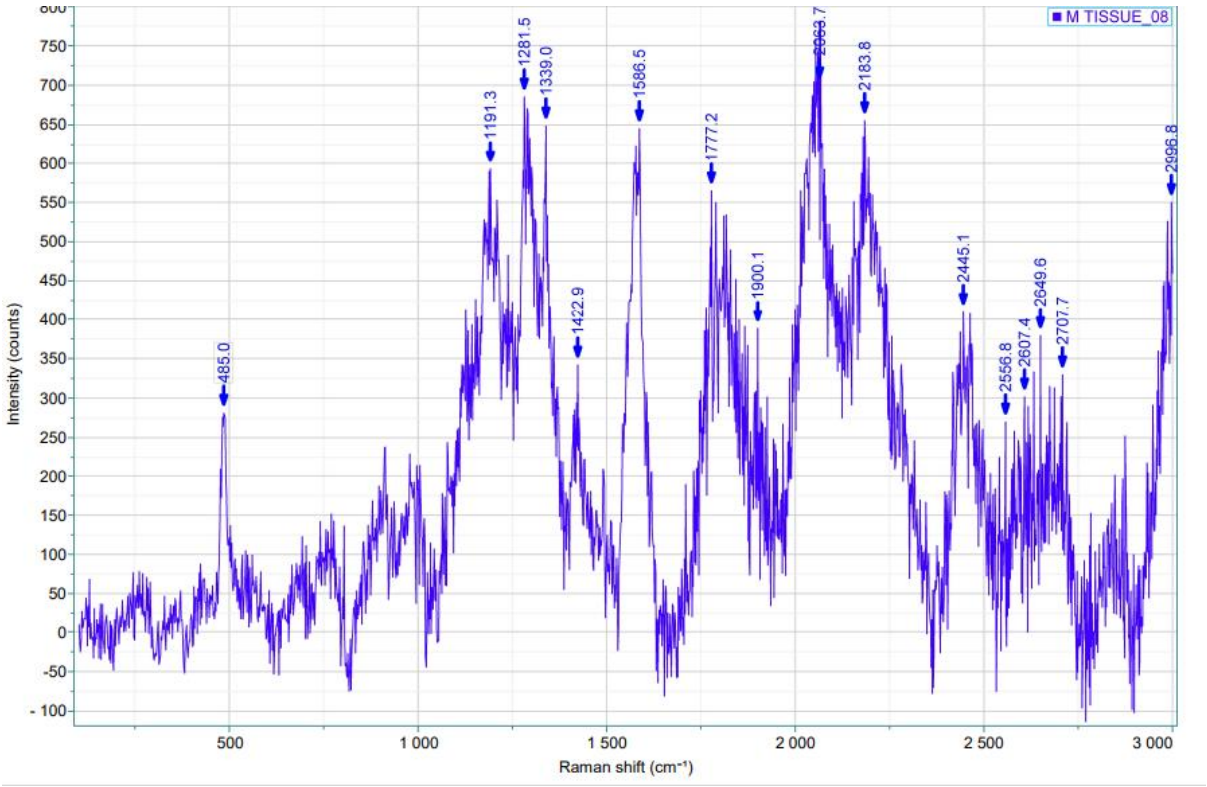


Fig 5 :- Raman Spectra of unknown microplastic identified as a Polyamide

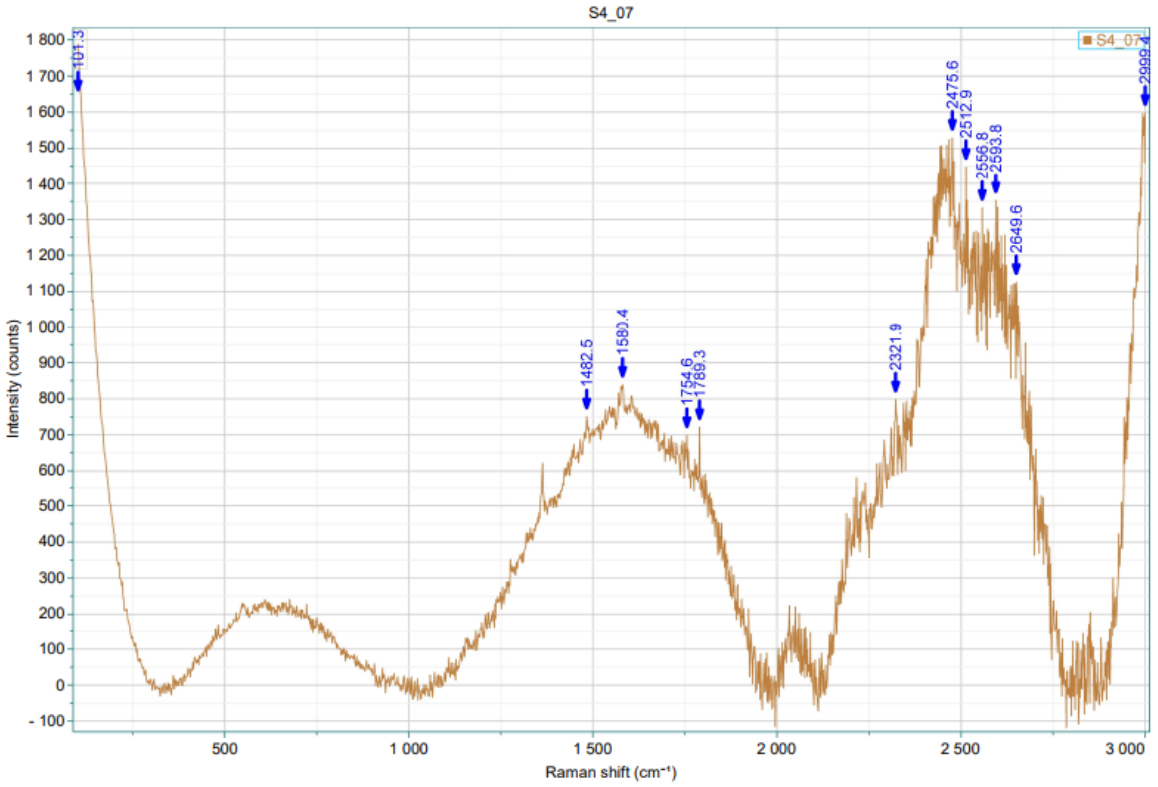


Fig 6 :- Raman Spectra of unknown microplastic identified as a Epoxy

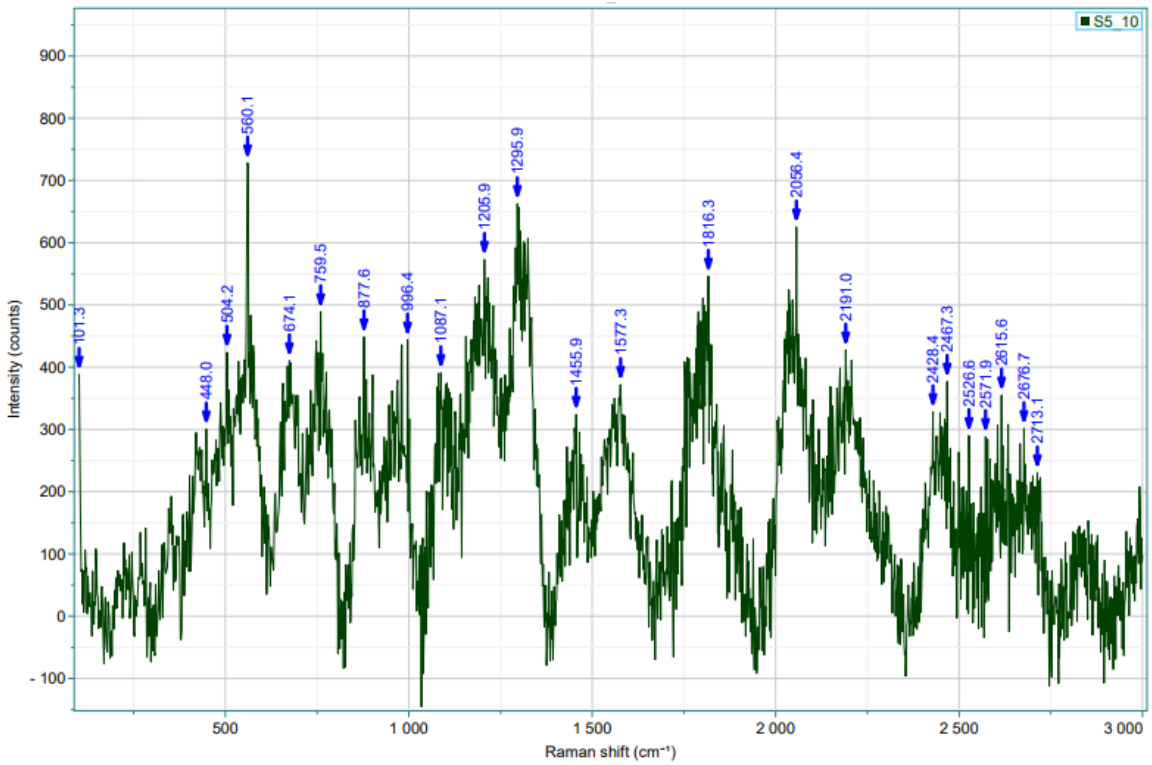


Fig 7 :- Raman Spectra of unknown microplastic identified as a Polyvinyl Chloride

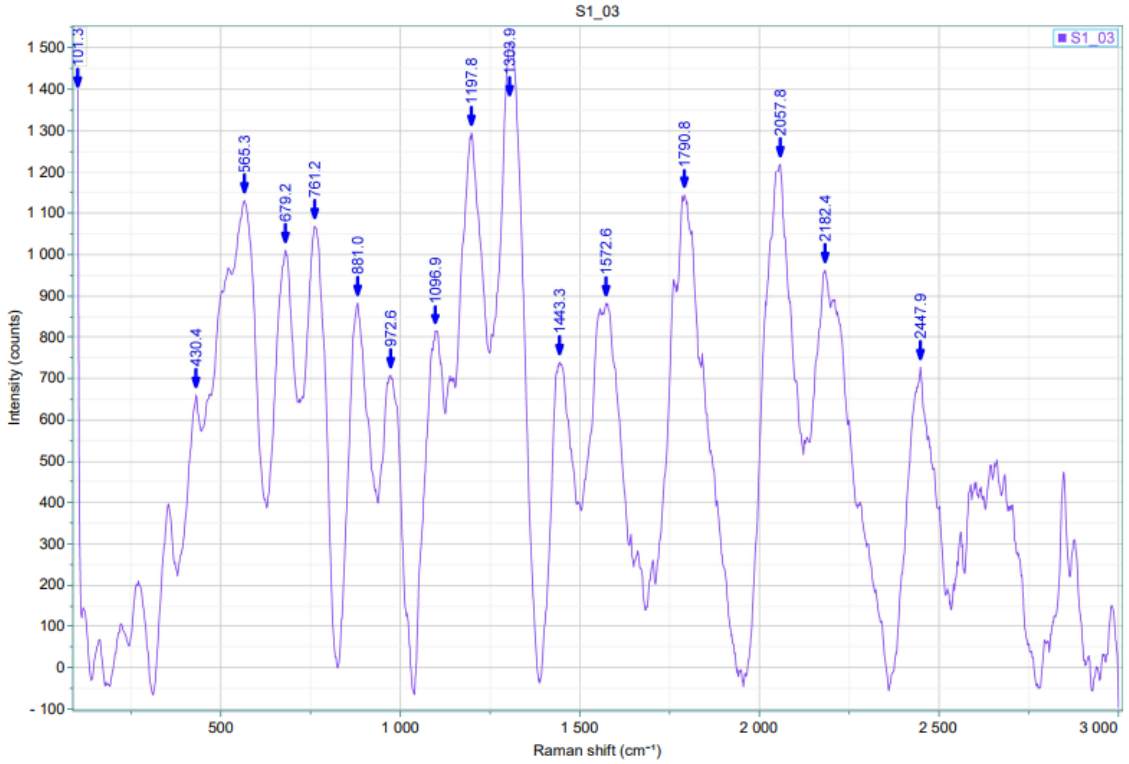


Fig 8 :- Raman Spectra of unknown microplastic identified as a Polyethyelene Terapthalate

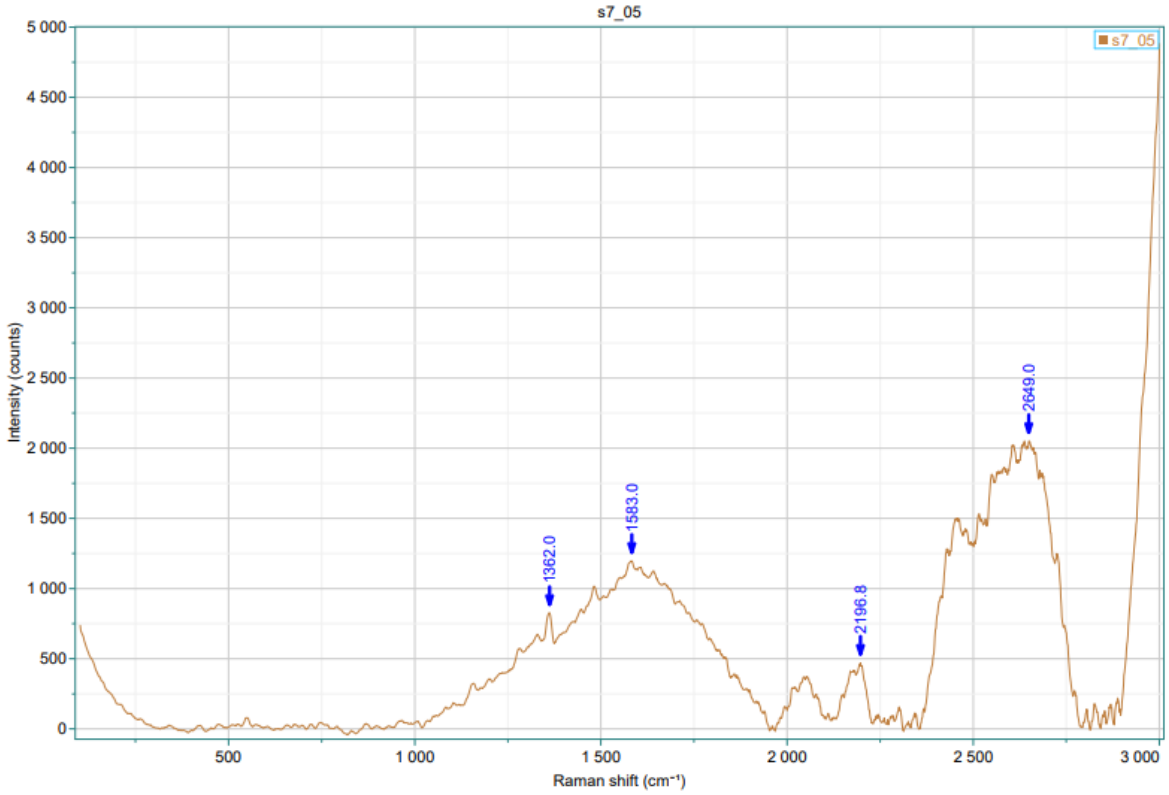


Fig 9 :- Raman Spectra of unknown microplastic identified as a Polyethyelene Vinyl acetate

PLATE -3

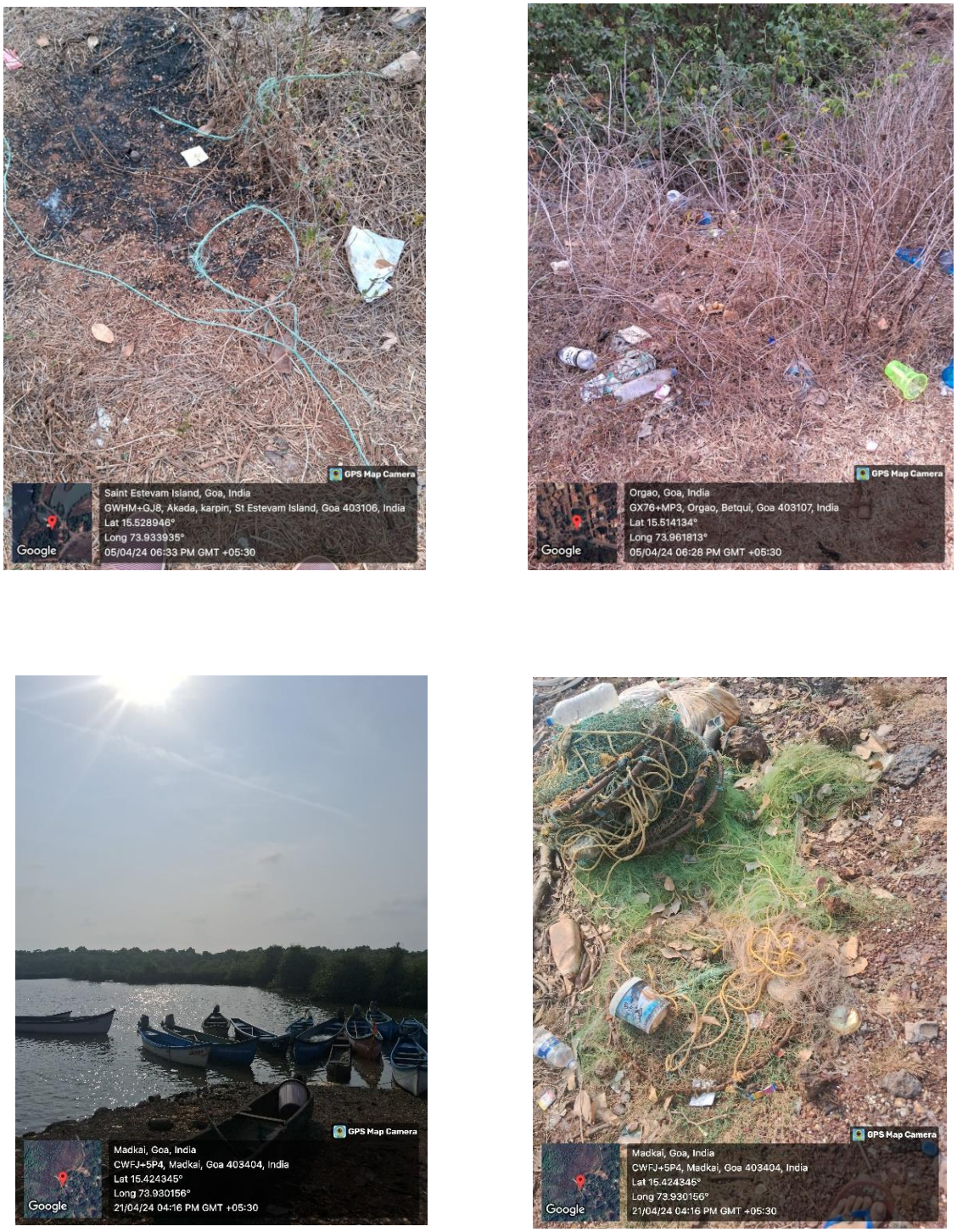
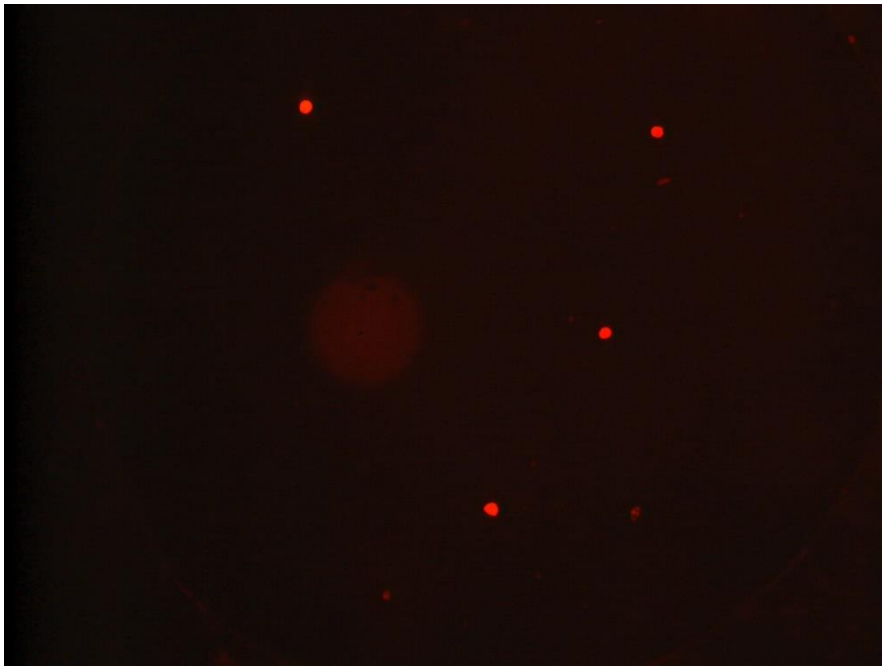


Fig 1:- Plastic pollution at selected study sites

PLATE -4

Genotoxicity test was peformed- only intact hemocytes were observed indicating that no DNA damage across the study sites



4.2 DISCUSSIONS

In the present study, MP's were found in the hepatopancreas of shrimp and water samples collected from the sluice gate harvest sites across a period of three months and biomarkers such as Condition Index, TBA assay, Catalase activity, ALP activity, ALT activity, Protein estimation and genotoxicity were evaluated. Microplastic were found to be accumulated in hepatopancreas of shrimps as well as found in the surface water of the sluice gate harvest sites across the three distinct sites that is Saint Estevam Island, Cundaim and Madkaim. Among the various sampling sites, Saint Estevam Island exhibited notably higher microplastic abundance, suggesting elevated pollution levels. This observation may be attributed to multiple factors, including extensive fishing activities, the discharge of domestic waste and sewage at the sluice gate, and harvesting activities at adjacent sites, indicating the temporal fluctuations in contamination levels across the studied sites.

Similar results were found of microplastic accumulation in the gastrointestinal tract of *Metapenaeus monoceros, Parapeneopsis stylifera,* and *Penaeus indicus,* at Arabian sea (Gurjar et al., 2021). Studies have also been carried out on seasonal variations in microplastic pellet (MPP) abundance along the Goan coast highlighting the significance of accounting for regional influences such as ocean currents and proximity to the pollution sources. They also discussed that the presence of fragmented microplastics found among recovered samples suggests that the predominant origin of microplastic contamination along the beaches of Goa comes from the degradation of larger plastic objects, leading to the formation of secondary microplastics (Veerasingam et al., 2016).

The accumulation of microplastics in shrimp tissues at study sites is likely facilitated by ingestion, as these particles resemble food items and similar findings were reported, indicating that

microplastic particles are ingested either directly, due to their resemblance to natural food items, or indirectly, through their adherence to food particles and the color of plastic items influences their bioavailability by enhancing their resemblance to natural prey, impacting their visual recognition (Ory et al., 2017). Walkinshaw et al. (2020) suggested that microplastic ingestion may not always signify adverse effects, as rapid egestion can occur through post-ingestion rejection or fecal expulsion. However, if microplastics or nanoplastics permeate tissues or the circulatory system via mechanisms like gut lining or gill transfer, they may accumulate and induce negative effects, posing challenges for removal by the organism.

Recent studies by Tong et al. (2024) have investigated how microplastics affect the exoskeleton of *L. vannamei* shrimp. Microplastics can disrupt the chitin structure, infiltrate the shells, and interfere with shell-building processes. These changes pose a threat to shrimp survival and marine ecosystems. Study has also been reported in Cochin, India, found microplastics in marine shrimp for the first time in *Fenneropenaeus indicus*, with higher contamination during the monsoon season. It highlights a potential route of human exposure to microplastics through consuming shrimp, especially when not deveined (Daniel et al., 2020).

In this study, fragment MPs were found to be abundant, followed fibers, pellets and film in hepatopancreas of shrimps at Saint Estevam Island and Cundaim. This is consistent with observations made by Yao, et al. (2021) and Chubarenko et al. (2016). Fibers were found to be more abundant in water sample at Saint Estevam island and Madkaim sites. Similar studies has been reported that fibers constitute the most prevalent form of microplastic pollution in marine waters globally (Walkinshaw et al., 2020). At Madkaim, the hepatopancreas of *L. vannamei* displayed fibers as the predominant form of microplastics, consistent with the findings of Hossain

et al. (2020), which also identified fibers as the dominant microplastic type in tiger shrimp, followed by particles and fragments.

The prevalence of fragment-shaped microplastics in shrimp hepatopancreas samples suggests potential ingestion of larger plastic debris by the shrimp, which may have broken down into smaller fragments within their digestive tract. Conversely, the predominance of fiber-shaped microplastics in water samples indicates a higher abundance of textile-related microplastics, possibly originating from land-based sources and transported via runoff or atmospheric deposition. Studies have suggested that microplastics entering the water system can be primarily sourced from cosmetic beads, industrial pellets, and personal care items like toothpaste, contributing to pollution (Wang et al., 2018). Furthermore, the observed variation in microplastic shapes between shrimp hepatopancreas and water samples across the three study sites could be attributed to differences in sources and transport mechanisms of microplastics.

The predominance of microplastics within the 1-50µm range in both shrimp hepatopancreas and water samples across the three study sites suggests a higher abundance of smaller particles. This could be attributed to the fragmentation of larger plastic debris into smaller fragments over time, facilitated by environmental factors such as UV radiation and mechanical abrasion. Additionally, smaller microplastics may be more readily transported and dispersed in aquatic environments due to their reduced size and increased surface area. These dynamics illustrate how both natural processes and human activities impact the distribution of microplastics in water. Studies have found that physical environmental factors contribute to the degradation of microplastics of different sizes in the environment, as the shrimps and crabs lack the enzymes necessary for breaking down synthetic materials (Andrady, 2021).

Microplastics are most commonly transparent, white, and blue. The dominance of blue microplastics across all sites indicates a prevalent source or widespread dispersion in marine environments, potentially originating from common consumer products. Transparent and white microplastics may stem from diverse sources like packaging materials and personal care items. Conversely, the smaller quantities of pink, red, and yellow microplastics suggest less frequent sources or limited usage compared to other colors. This variation underscores the diverse origins and types of microplastics in aquatic ecosystems, highlighting the intricate nature of plastic pollution. Similar findings were observed in the Australian glass shrimp, *Paratya australiensis*. The predominant color observed in both water and shrimp samples from Australian waters was blue. Previous studies by Saha et al. (2021) and Yao et al. (2021) reported similar color patterns were recorded such as transparent microplastics were the most abundant, comprising the majority of all microplastics and Blue, green, white, and yellow microplastics were also common. Microplastic samples displayed discoloration at study sites, consistent with findings from Oktafira et al. (2021), indicating a color transition from blue to transparent in certain fibers. This suggests potential prolonged exposure in coastal waters and oxidation over time.

The colored microplastics (MPs) observed in our study likely originated from common items such as clothing, disposable plastic bags, and commodity packaging. Plastic pollution was observed at selected sites as shown in plate 3. Conversely, the transparent MPs may have originated from fishing gear such as nets and fishing lines, as suggested by Kalangutkar et al. (2024) findings.

The analysis and validation of various polymer types using μ-Raman Spectrophotometer provided insights into the composition of microplastics found in both shrimp hepatopancreas and water samples across three sites. Identified polymers such as Polyethylene Terephthalate (PET),

Polyamide, Polycarbonate, Poly Vinyl Chloride (PVC), Polyacrylonitrile, Polyethylene Vinyl Acetate, Epoxy, Thermoplastic polyester and Polyethylene highlight the diverse range of plastic materials present in the environment. Research findings by Schymanski et al. (2018) indicates the presence of microplastic fragments in water samples, the dominant polymers identified were polyester (primarily PET) and polypropylene, indicating the possibility of microplastic leaching from packaging materials. Raman Spectroscopy analysis confirmed the dominant presence of polyethylene terephthalate (PET), polypropylene (PP), and polystyrene (PS) as the polymer types in microplastics (MPs). MPs were detected in the digestive tissues of white shrimp (*Metapenaeus affinis*). Polymer analysis conducted by Gurjar et al. (2021) indicates that microplastic pollution in the studied area predominantly originates from sources such as laundry and domestic wastewater, fishing gears, and food packaging materials. PET is commonly utilized in textiles such as clothes, blankets, and fleeces (Wang et al., 2017a, 2017b). In contrast, PE and PP are extensively employed in packaging bags, fishing nets, and agricultural films due to their stability and lightweight characteristics (Liu et al., 2020; Wang et al., 2019).

The Coefficient of Microplastic Impact (CMPI) serves as a crucial metric in assessing the ecological repercussions of microplastic contamination in aquatic ecosystems. The research findings suggest that microplastic pollution, characterized by varying shapes such as fibers, pellets, fragments, and films, exhibits differential impacts on aquatic organisms and environments across distinct sites such as Saint Estevam Island, Cundaim, and Madkaim. The observed moderate to substantial impacts indicate a concerning trend, potentially influenced by local anthropogenic activities, hydrodynamic conditions, and waste management practices. Kalangutkar et al. (2024) revealed in their study along the Chapora River that the morphology of microplastics dictates their impact coefficient (CMPI). Fibers exerted the greatest influence at various sites, attributed to

human activities. Fragments showed high impact at one site and moderate impact at other sites. Films showed moderate impact at three sites and minimal impact at other locations.

The increasing prevalence of microplastics poses significant threats to human health and the environment, necessitating prompt measures for detection and removal to prevent bioaccumulation. Strategies for microplastic removal, including filtration, surface adhesion, and deterioration, offer potential solutions and merit further exploration in mitigating this environmental concern suggested by Pandey et al. (2022). Eamrat et al. (2024) proposed chitosan, derived from shrimp shells, as an effective and eco-friendly solution for removing microplastics from water. Their study found that combining chitosan with alum led to the formation of larger particles, enhancing microplastic removal efficiency. This approach involves neutralizing microplastic charges and forming larger flocs for easy removal from water bodies. The detection of microplastics in shrimp highlights concerns about their potential accumulation and transfer within the food chain. The identification of microplastics underscores the necessity to develop strategies aimed at their removal and mitigation.

The comet assay, a DNA damage detection method, found no comet formations, indicative of healthy cellular DNA. Additionally, only intact hemocytes were observed. The absence of genotoxicity despite microplastic presence in shrimps across the sites could be due to variables like microplastic size, composition, degradation status, and the shrimp's internal detoxification mechanisms.

Limited studies have been conducted on the genotoxicity of microplastics on shrimps. One such study revealed that shrimps when exposed to polystyrene microspheres concentrations of 40 and 400 µg/kg exhibited genotoxic effects. Significant DNA damage was found in the hepatopancreas

of shrimps exposed to 400 µg MP/kg. Conversely, those exposed to 40 µg MP/kg showed reduced DNA damage in both muscle and hepatopancreas compared to other groups, indicating that exposure to microplastics can induce DNA damage, impacting genetic information transmission and ecological balance (Seta et al., 2023). Also similar studies on freshwater shrimp *Neocaridina davidi* revealed the potential DNA damage in the shrimp (Berber, 2019).

Shrimp emerges as a prime protein source, with 19.4g of protein per 100g and accounting for 87% of its total energy. Proteins are continually being used by the animal for growth and repair of tissues. Shrimps play a vital role in providing essential amino acids. With most of its edible portion water and nearly 80% of the rest packed with protein, shrimp emerges as an exceptional option for meeting dietary requirements (Dayal et al., 2013).

The significance of the protein content in the present study lies in its unique procurement from distinct sluice gates, each representing a specific site with its own environmental characteristics. These site differences offer a valuable opportunity to investigate how variations in environmental conditions, dietary habits, and other local factors influence protein levels in muscle tissue. Therefore, the protein content analyzed in this study is significant as it reflects the diverse conditions and contexts of the different sluice gates. The studies have revealed that optimal dietary protein requirements for whiteleg shrimp (*L. vannamei)* vary depending on factors such as shrimp size, body weight, culture system, stocking density, environmental conditions, protein source quality, non-protein energy, culture salinity, and temperature (Shahkar et al., 2014; Brito et al., 2001).

Antioxidant enzymes serve as vital biomarkers of oxidative stress, tasked with eliminating ROS and pro-oxidants from cells. Superoxide dismutase (SOD), catalase (CAT), glutathione

peroxidases (GPX), glutathione reductase (GR), Thiobarbituric Acid Reactive Substances (TBARS) assay and glutathione-S-transferase (GST) are key antioxidant enzymes, pivotal in combating oxidative stress. Reactive oxygen species (ROS) are highly reactive molecules, damaging cell membranes, proteins, enzymes, and DNA, leading to tissue and organ dysfunction. Control of ROS levels is essential for cellular health and preventing oxidative stress-related damage (Halliwell, 2006; Li et al., 2016). Lipid peroxidation, resulting from ROS reacting with lipids, is a common form of cellular damage and a key biomarker of oxidative stress. Malondialdehyde (MDA) levels, reflecting the extent of lipid peroxidation, serve as a crucial indicator of oxidative state in samples (Lesser, 2011).

The analysis of catalase enzyme estimation reveals significant results influenced by various factors, while the investigation into lipid peroxidation levels, evaluated via the TBARS assay, indicates an insignificant interaction between months and sites. This suggests that average outcomes are influenced by both temporal (months) and spatial (sites) factors. The presence of microplastics may induce oxidative stress in shrimps, leading to significant alterations in enzyme activity and lipid peroxidation levels. Similarly, Xing et al. (2014), Gholamhosseini et al. (2024), and Seta et al. (2023) explored the impact of microplastic (MPs) exposure on various shrimp tissues, emphasizing the induction of oxidative stress. These studies contribute to understanding the broader implications of environmental pollutants on aquatic organisms and underscore the importance of addressing oxidative stress in assessing ecosystem health.

AST, ALP and ALT activities are widely recognized as general markers for tissue damage. (Santhoshkumar et al., 2017) ALT, prevalent in animal hepatocytes like shrimps, is pivotal in protein metabolism and serves as a common biomarker for assessment (Fabrello et al., 2022).

Conversely, ALP, found in multiple tissues including hepatopancreas and gills, contributes to various physiological functions such as nutrient metabolism and immune response, with elevated levels indicating tissue damage and cellular stress (Fernandez and Kidney, 2007).

Research conducted by Gholamhosseini et al. (2024) explored the individual and combined impacts of microplastics and lead acetate on freshwater shrimp (Caridina fossarum), revealing hepatotoxicity through elevated ALP, AST and ALT levels, indicating biochemical effects and physiological responses. Significant variations were observed in Alanine Transferase (ALT) and Alkaline Phosphatase (ALP) levels in shrimps across different sites and months, indicating potential hepatotoxicity and environmental stressors. Tukey's multiple comparisons test highlighted significant differences in ALT and ALP levels among sites over the study period suggesting potential hepatotoxicity and environmental stressors.

Condition factor (K) serves as an index reflecting the physiological well-being of aquatic organisms like shrimp, integrating both biotic and abiotic factors. It evaluates the species' energetic condition and reserves based on weight at a given length (Komi and Francis, 2017). In the present study conducted it reveals a significant interaction effect between months and sites in the condition index analysis. However, neither the main effect of months nor the main effect of sites alone is significant, suggesting differences among the sites. These condition index variations could be because of different geographical locations and availability of food.

CONCLUSION

The present study investigated the accumulation of microplastics in water and hepatopancreas of shrimp L. vannamei across different sites. Parameters including size, shape, color, abundance, and coefficient of MP shape were measured and observed. Validation was done by μ-raman spectroscopy. Genotoxicity assessment revealed no damage to hemocytes from MP pollution in water. Physiological and biochemical parameters such as catalase, TBA, ALT, ALP, and protein in muscle and hepatopancreas were evaluated, showing significant variations across sites. Furthermore, condition index of all sampled shrimp differed, indicating potential impacts of environmental factors on their health and well-being. This comprehensive analysis provides valuable insights into the ecological impact of MP pollution on shrimp populations, highlighting the need for continued monitoring and mitigation efforts to safeguard aquatic ecosystems and biodiversity.

To effectively address microplastic pollution, several crucial steps must be taken. Firstly, implementing strict regulations to mitigate microplastic pollution, secondly, fostering public awareness campaigns on responsible plastic usage. Also conducting regular monitoring of water bodies, and further researching the long-term effects of microplastic exposure on aquatic organisms. The study's multi-site approach offers a foundation for future research to expand into broader geographical regions, facilitating a more comprehensive understanding of microplastic distribution and its impact on diverse ecosystems. Future investigations may explore microplastics' interactions with other pollutants and aquatic organisms.

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