Evaluation of the Toxic Effects of Polystyrene Microplastics in Tilapia

(*Oreochromis mossambicus*)

by

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I hereby declare that the data presented in this Internship report entitled, "Evaluation of the Toxic Effects of Polystyrene Microplastics in Tilapia (*Oreochromis mossambicus*)" 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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PREFACE

Concerns about the widespread existence and harmful impacts of microplastics have grown in the field of aquatic ecology. Polystyrene microplastics are one of these artificial invaders that are particularly noticeable due to their extensive occurrence and possible harm to aquatic life. As polystyrene microplastics infiltrate into their environments, tilapia—a crucial species in aquaculture and freshwater ecosystems, will be subjected to a growing amount of these particles. These microscopic plastics are capable of upsetting the physiological functions and ecological dynamics of tilapia populations in spite of their small size.

The context for an investigation into the complex impacts of polystyrene microplastics on tilapia is established by this introduction. We seek to clarify the methods by which these microplastics affect the behaviour, health, and population dynamics of tilapia through thorough research and scientific inquiry.

We must investigate the specifics of this problem as stewards of aquatic environments and work to reduce the negative impacts of polystyrene microplastics on tilapia and their ecosystems. To successfully complete this project, interdisciplinary cooperation, creative research, and a dedication to preserving the integrity and well-being of our freshwater ecosystems and the animals that live there are necessary.

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ABSTRACT

Although microplastics (MPs) have been identified in marine ecosystems globally, attention has increasingly focused on the significance of MPs pollution in freshwater environments in recent years. Nevertheless, our understanding of the biological impacts of MPs on freshwater organisms remains considerably less extensive compared to marine organisms. The aim of the present study was to evaluate the accumulation and tissue distribution of MPs in the freshwater fish Mozambique Tilapia (*Oreochromis mossambicus*), as well as the biochemical and genotoxic effects of MPs on *O. mossambicus*. During 14 days exposure of Polystyrene (PS) microplastics at 50mg/L, 250mg/L and 500mg/L concentrations were exposed to *O. mossambicus*. Concentrations in various tissues of *O. mossambicus* generally increased with time in concentration dependent manner following the order gill>gut>liver. Fish exposed to PS-MPs displayed histopathologic alterations in the gut and gill tissues. The exposed fishes showed significant difference in enzymatic concentrations such as Catalase, Thiobarbituric Acid Reactive Substances, Alkaline Phosphatase and Alanine Transaminase. No significance was observed in Protein and Carbohydrate levels in the experimental groups. Additionally Polystyrene miocroplastics significantly increased the number of micronucleated cells in exposed fish erythrocytes showing chromosomal damage, on the other hand negative results were found for DNA break damage tested by comet assay. These findings underscore the ingestion and buildup of microplastics (MPs) in various tissues of freshwater fish, resulting in disruptions to fish biological systems. This factor should be taken into account in environmental risk assessments.

CHAPTER 1

INTRODUCTION

1.1. MICROPLASTICS

Microplastics are synthetic compounds characterized by a high molecular weight that has undergone reduction, resulting in plastic particles smaller than 5 mm. this process proceeds in the following ways: conditional film formations, colonization, bio-fragmentation, assimilation, and mineralization (Corcoran, 2019). Due to their slow biodegradation, these particles persist in the environment, adversely affecting human health, especially for individuals who consume them as the last step in the food chain. They are prevalent pollutants in diverse environments, including oceans, remote islands, and Polar Regions, posing a growing threat to ecosystems (Cole *et al.*, 2011). The harmful effects of microplastics prompt their classification based on source and size of fragmentation, broadly categorized as primary and secondary. Primary microplastics are intentionally created particles in consumer products like detergents and cosmetics, while secondary microplastics stem from products that contain plastic, such as plastic waste, fibres, or plastic items that have broken down after being exposed to the environment (Wang *et al.,* 2019).

These minute plastic particles can take various forms, including fragments, fibres, beads, and foam, and they are found in the environment as a result of plastic pollution and the breakdown of larger plastic items, such as plastic bottles, plastic bags, plastic utensils, etc. These particles can persist as marine pollutants for as long as 450 years (Hopewell *et al.,* 2009).

Approximately 12 million metric tons of plastic find their way into the ocean annually. Over time, this plastic waste disintegrates into smaller fragments, referred to as micro and nanoplastics (Eunomia, 2016). Although less conspicuous, these minute particles can have severe repercussions on marine ecosystems and potentially jeopardize the Earth's climate stability. Recent estimates propose that around 358 trillion microplastic particles float on the ocean's surface, and even more exist in its deeper realms. Their prevalence raises concerns due to the likelihood of marine life mistaking them for food. From colossal filter-feeding whales to tiny plankton at the foundation of the food chain, a variety of species may inadvertently ingest these microscopic plastic particles (Asher, 2023).

1.2. MICROPLASTICS IN AQUATIC ECOSYSTEM

Microplastics are an emerging pollutant that invades different environments via different sources; the material enters the marine environment through terrestrial and land-based activities, especially via run-offs, and is known to have a great impact on marine organisms (Auta *et al.*, 2017). Microplastic studies in aquatic ecosystems are essential because plastic degradation results in minuscule particles, presenting environmental risks. Aquatic environments, being interconnected and delicate, act as vital indicators of the extensive influence of microplastics on ecosystems, wildlife, and potentially human health through the food chain (Harrison *et al.*, 2011). Investigating the occurrence, dispersion, and impacts of MPs in water bodies aids in deriving strategies for mitigation and conservation. Microplastics enter the marine ecosystem through various sources which include fishing gear (lost, discarded, or abandoned) tourism, sewage systems, drainage, mismanaged disposals, littering by the beachgoers, etc. Microplastics in marine sediments can disturb microbial communities and interfere with nitrogen cycling. This may escalate existing human-induced issues such as toxic algal blooms. Alterations in surface ocean plankton communities have the potential to worsen deoxygenation linked to climate change, leading to oxygen deprivation for marine organisms (Asher, 2023).

As plankton-contaminated faeces and bodies, laden with microplastics, sink, they transport plastic particles from the surface to the deep ocean. This phenomenon may impact seafloor organisms essential for nutrient cycling, moving us towards a potential breach of another planetary boundary—biogeochemical nitrogen flows (Hitchcock, 2022).

Research indicates that when exposed to elevated concentrations of microplastics, phytoplankton may change in photosynthesis (Mao *et al.*, 2018; Wu *et al.*, 2019), while zooplankton may experience a decrease in feeding rates. Furthermore, studies have established a connection between the ingestion of microplastics by these organisms and adverse effects on growth, lifespan, reproduction, and fertility within various plankton species. These repercussions can potentially cascade through the entire food chain.

Microplastics can also absorb other pollutants and trace metals present in the marine environment becoming more harmful (Cole *et al.*, 2011). Microplastics with a diameter of fewer than 130µm have the potential to translocate into human tissue, elicit a localized immune response, and release constituent monomers, toxic chemicals added during plastic production, and pollutants absorbed from the environment, such as heavy metals and various organic pollutants like PCBs and DDT (Wright *et al.*, 2017). Biomagnification and bioaccumulation of such pollutants at the trophic level

will have detrimental effects on the health of higher organisms which can lead to infertility, obesity, and cancer (Rochman *et al.,* 2013).

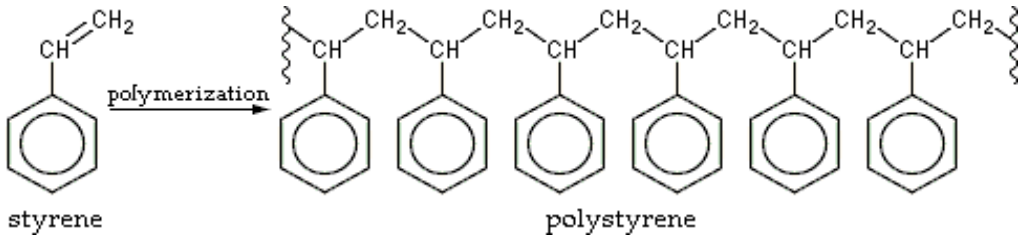
1.3. MICROPLASTIC IN FRESHWATER ECOSYSTEM

Plastic pollution has become a widespread global issue, posing significant challenges for environmental protection and the management of aquatic resources (Wong *et al.,* 2020). Unlike the open marine environment, freshwater systems are relatively enclosed, leading to the accumulation of microplastics due to slower flow rates, limited diffusion, and closer proximity to human activities (Andrady, 2011).

Various factors affect the distribution of microplastic, including large-scale phenomena such as wind-driven currents, geostrophic circulation (Law *et al.,* 2010), turbulence, and oceanographic processes (Ballent *et al.,* 2012; Turra *et al.,* 2014). Key factors such as the density, shape, and size of MPs greatly influence their movement and dispersion patterns (Medrano *et al.,* 2015). These factors are particularly influential in vast freshwater ecosystems such as river systems. Conversely, smaller, isolated freshwater bodies are more affected by natural elements and prolonged water residence times, which primarily govern microplastic levels (Free *et al.,* 2014). Various pathways contribute to the introduction of plastic into freshwater ecosystems, including land-based sources, fishing, shipping industries, soil erosion, run-off from terrestrial areas, and wastewater discharge (Lambert *et al.,* 2014). Once introduced, microplastics may either float freely in water bodies or settle in sediments, posing risks to aquatic organisms through ingestion and subsequent impacts will be apparent on growth, development and immune responses. This will not only affect the aquatic communities but also indirectly influences whole ecosystem functions by altering habitat properties (Porta *et al.,* 2023). Consequently, microplastic in dynamic freshwater environments often migrates to marine ecosystems, while those in stagnant water bodies tend to accumulate locally. Despite the extensive focus on microplastics in seawater, less that 4% of studies have investigated microplastic pollution in freshwater systems (Lambert and Wagner, 2018). This concern has prompted increased monitoring efforts by researchers and environmental experts to assess the impacts of plastics on ecosystem and wildlife.

1.4. POLYSTYRENE

Polystyrene is a polymer known for its transparency, rigidity, brittleness, and moderate strength in its unmodified form. It is produced through polymerization, involving the combination of the building block compound known as styrene. From a chemical perspective, polystyrene can be described as an extended hydrocarbon chain where carbon atoms alternate with phenyl groups, which are derivatives of benzene. Its chemical formula, $(C_8H_8)n$, indicates the presence of carbon and hydrogen elements within its structure. The characteristics of the material are dictated by the short-distance van der Waals forces operating between the polymer chains. As these molecules comprise thousands of atoms, the collective attractive force among them becomes significant (Scheirs & Priddy, 2003).



https://en.wikipedia.org/wiki/File:Polystyrene_formation.PNG

Each carbon within the backbone exhibits tetrahedral geometry, while those bonded to phenyl groups (benzene rings) are stereogenic. When the backbone is arranged as a flat, elongated zig-zag chain, each phenyl group is angled either forward or backward relative to the chain's plane. The sequential arrangement of phenyl groups dictates the tacticity, impacting a variety of physical properties of the material.

Polystyrene (PS) plays a vital role in contemporary plastic manufacturing; however, its extensive utilization and direct disposal into the environment significantly impact the food chain (Siddiqui *et al.,* 2023). Polystyrene finds application in various forms, including foam, film, and rigid plastic, used in diverse fields from food packaging to protecting electronics (Goyal *et al*., 2023). Despite its flexibility and low shrink rate, it has a slow flow and can become brittle. While polystyrene is considered food-safe, there is a potential for the leaching of styrene over time. It is generally deemed safe until issues like shedding or the migration of styrene, in the form of micro or nanoparticles, become apparent. Numerous research studies have established a link between styrene exposure and an increased risk of cancer, particularly through the ingestion of styrene

molecules leaching from food and drink containers (Cohen *et al.,* 2002; Lickly *et al.,* 1995a; ATSDR 2010).

Identifying the presence of this emerging pollutant in environmental components and comprehending its impact on aquatic ecosystems is crucial in researching its toxic effects, as well as the risk it poses to human populations in contact with contaminated water or consuming fish from affected water bodies. Currently, the lack of sufficient knowledge has led to the exclusion of allowable pollutant levels from water quality standards and genotoxicity studies. Therefore, there is a pressing need to investigate its presence, toxic doses, and associated problems to develop proposals for improved plastic waste disposal methods.

1.5. MODEL ORGANISM: TILAPIA

Kingdom **:** Animalia

Phylum **:** Chordata

Class **:** Actinopterygii

Order **:** Cichliformes

Family **:** Cichlidae

Genus **:** *Oreochromis*

Species **:** *O. mossambicus*

Fish has become a viable model organism in biomedical research, serving as a valuable platform for experimental pharmacological and toxicological studies (Harikumar *et al.,* 2022). Being a vertebrate, it possesses numerous conserved physiological and molecular characteristics similar to humans. This renders it a valuable model for diagnosing and investigating disease states, as well as testing the toxicity and therapeutic efficacy of drugs, pollutants, and other compounds against the intended targets (Hilgers *et al.,* 2019).

The Mozambique tilapia "(*Oreochromis mossambicus*)", characterized by its subdued hues, typically has a lifespan of about ten years in its original environment. It has become a favoured choice for aquaculture, having been introduced by humans to various tropical and subtropical regions worldwide. Its ability to thrive in different environments has led to concerns about its potential invasiveness. Nevertheless, this adaptability also renders it a desirable species for aquaculture ventures due to its capacity to acclimate easily to varying conditions.

Oreochromis mossambicus has been extensively distributed globally for aquaculture purposes and recreational fishing, hence it is a great medium to analyze accumulation, biomagnification, and related health risks. One notable benefit of tilapias is their capability to thrive on plant-based diets, although their inefficient plant digestion due to a lack of cellulase is a limiting factor (Towers, 2015).

Due to its fast growth, relatively small size when it reaches sexual maturity, good adaptability to different environmental conditions, and economic importance, *O. mossambicus* is an excellent experimental model. The presence and effects of microplastics in tilapia provide a solid foundation for risk assessment in humans (Bhuyan, 2022). It also helps in the assessment of environmental risk and hazardous concentrations due to the high tolerance level of tilapia for harsh conditions. This particular organism can serve as a model for various species within aquatic ecosystems, particularly those consumed by humans, in terms of assessing the toxic effects of pollutants.

The impact of microplastic on human health remains inadequately comprehended. Given their prevalence in the environment, exposure can happen through ingestion, inhalation and skin contact. The harmful repercussions of microplastics on both fish and human beings have yet to be fully elucidated. This detailed report which hypothesizes that the Polystyrene microplastics induce toxic effects in fish, through examination could contribute valuable insights into the eco-toxicological impacts of microplastics on both fish and humans, thereby aiding future research endeavours.

1.6. BACKGROUND OF THE PROBLEM

Despite the considerable advantages plastics offer society, our reliance on them comes with drawbacks. One such growing concern is the build-up of plastics in the aquatic environment. Among the numerous human impacts on aquatic environments, the build-up of plastic waste stands out as both conspicuous and under-researched. Factors such as their long-lasting nature, unsustainable consumption, and inadequate disposal methods contribute to a significant accumulation of plastics in natural settings. Given the common routes through which plastic enters the ocean, it is imperative to recognize the freshwater system's significant role in the broader plastic pollution issue due to its direct link to human existence. (Kumar *et al.*, 2022).

1.7. LACUNAE

Although the research on marine microplastics (MPS) is well-developed, significant knowledge gaps exist regarding freshwater MPs. Information regarding their absorbance is incomplete for larger water bodies and entirely lacking for smaller surface waters. Additionally, there is a need to investigate relevant sources and the environmental fate of freshwater microplastics. Furthermore, data on the biological impacts of microplastics on freshwater species is notably lacking. The accumulation of other contaminants of microplastic in freshwater environments is particularly intriguing due to the potential for increased chemical exposure through ingestion. However, data on such crucial issues remains unavailable.

1.8. OBJECTIVES

- To determine the acute toxicity of polystyrene microplastic in Tilapia (*O. mossambicus*).
- To detect the accumulation of polystyrene microplastic in different tissues in Tilapia (*O. mossambicus*).
- To evaluate the genotoxicity induced by polystyrene microplastic in Tilapia under laboratory exposure conditions.

CHAPTER 2

REVIEW OF LITERATURE

Chaoran Li and colleagues conducted a comprehensive assessment on the presence of microplastics in freshwater ecosystems. Their findings reveal that the primary sources of microplastics in freshwater environments include synthetic textiles, personal care products, industrial raw materials, and improper disposal of plastic waste. They emphasized that microplastic pollution spans a wide range of concentrations, underscoring its status as a global concern. The predominant polymer constituents of microplastics in freshwater were identified as polyethylene (PE), polypropylene (PP), polystyrene (PS), and polyethylene terephthalate, collectively constituting 70% of the total, each with similar frequencies of occurrence. Overall, this review contributes significantly to our understanding of the scope and impact of microplastic pollution in freshwater ecosystems, underscoring the urgent need for effective mitigation strategies (Li *et al.*, 2020).

The review article "Microplastics in Freshwater Environments: Sources, Fates, and Toxicity" by Ding, Tong, and Zhang provides a comprehensive overview of the presence, origins, behaviours, and impacts of microplastics in freshwater ecosystems, highlighting the diverse pathways through which these pollutants enter freshwater environments. Furthermore, the article explores the fate of microplastics in freshwater systems, including their transport, deposition, and accumulation in different environmental compartments. One of the strengths of this review is its discussion on the toxicity of microplastics to aquatic organisms (Ding *et al.*, 2021).

Peng *et al.*'s (2018) study on microplastics in freshwater river sediments in Shanghai offers a compelling insight into the environmental challenges facing mega-cities. Through detailed analysis, the research underscores the prevalent presence of microplastics in urban water systems, shedding light on the potential risks they pose to ecosystems. The study's risk assessment framework provides valuable guidance for policymakers and environmental agencies in managing microplastic pollution. By focusing on a rapidly urbanizing area like Shanghai, the research underscores the urgent need for comprehensive strategies to mitigate microplastic contamination in similar urban settings worldwide. However, further investigation into the sources and impacts of microplastics in freshwater ecosystems could enhance the study's depth and applicability.

The assessment by Vanapalli *et al.* (2021) offers valuable insights into microplastic pollution in aquatic ecosystems from an Indian perspective. The study provides a comprehensive examination of the prevalence and impacts of microplastics, shedding light on the environmental challenges faced in the region. The study emphasizes the necessity of focused approaches to reduce microplastic contamination in Indian waterways. By focusing on a specific geographical context,

the study enhances our understanding of the global issue of microplastic contamination. The findings contribute to the growing body of literature on microplastics, informing future research directions and management efforts.

The study by Neelavannan *et al.* (2022) presents a significant assessment of microplastic contamination in freshwater lake sediments in the high-altitude Himalayas, specifically in the Northwest Himalayan region of India. The research highlights the presence and extent of microplastic pollution even in remote and high-altitude environments. This study focuses on the global reach of microplastic pollution, emphasizing its penetration into pristine ecosystems. Even in remote areas like the Himalayas, the results highlight the critical need for all-encompassing initiatives to reduce microplastic pollution.

Veerasingam *et al.* (2016) conducted a study investigating the characteristics, seasonal distribution, and surface degradation features of microplastic pellets along the Goa coast in India. Their research provides valuable insights into the abundance and dynamics of microplastics in a coastal environment subjected to varying seasonal conditions. By examining surface degradation features, the study offers important information on the weathering processes affecting microplastic particles in marine ecosystems. This study contributes to our understanding of microplastic pollution in Indian coastal regions and informs efforts to protect marine environments.

Saha *et al.* (2021) presented a compelling case study on the emergence of microplastics in seafood as a significant threat to the marine environment, focusing on the west coast of India, particularly in Goa. Their study illuminates the widespread occurrence of microplastics in seafood, emphasizing the potential threats they pose to both aquatic ecosystem and human health. By conducting thorough analysis, the research emphasizes the immediate necessity for increased awareness and implementation of management strategies to tackle this escalating issue. By focusing on a specific geographical area and food source, the research provides valuable insights into the local impacts of microplastic pollution on marine resources. Saha et al.'s work serves as a critical contribution to understanding the complex interactions between microplastics, marine life, and human consumption, urging for comprehensive strategies to mitigate this emerging threat to coastal environments.

Scherer *et al.* (2018) provide a comprehensive examination of the interactions between microplastics and freshwater biota in their chapter within the handbook "Freshwater

Microplastics." Their work synthesizes existing research to elucidate the various ways in which microplastics affect freshwater organisms and ecosystems. By exploring the impacts on different levels of biological organization, from individual organisms to entire food webs, the chapter offers valuable insights into the ecological consequences of microplastic pollution in freshwater environments. Through a multidisciplinary approach, including ecotoxicology, behavioural ecology, and physiological studies, the authors highlight the complexity of microplastic-biota interactions. This chapter serves as an essential resource for scientists, policymakers, and stakeholders seeking to understand and address the challenges posed by microplastic contamination in freshwater systems.

Zhang *et al.*'s (2017) study investigates the occurrence and characteristics of microplastic pollution in Xiangxi Bay of the Three Gorges Reservoir in China. The study's thorough analysis highlights the serious environmental threat that plastic contamination in reservoirs poses by demonstrating the pervasiveness of microplastics in this freshwater ecosystem. The study provides valuable insights into the types and sources of microplastics found in the bay, contributing to our understanding of the pathways of plastic contamination in large water bodies. By highlighting the extent of microplastic pollution in the Three Gorges Reservoir, the research underscores the importance of implementing effective management strategies to mitigate further contamination and protect aquatic ecosystems.

Pandey *et al.* (2023) conducted a study on the abundance, characteristics, and risk assessment of microplastics in indigenous freshwater fishes of India. Their research provides critical insights into the prevalence and potential impacts of microplastic pollution on freshwater fish species native to India. The study sheds information on the extent of contamination and associated hazards by analyzing the microplastics' properties and quantity in the gastrointestinal tracts of these fish. By assessing the potential harm posed by microplastics to fish health and ecosystem integrity, the research underscores the urgent need for mitigation measures. This study serves as a valuable resource for policymakers, conservationists, and scientists working towards the preservation of freshwater biodiversity in India.

Daniel *et al.* (2020) investigated the abundance, characteristics, and seasonal variation of microplastics in Indian white shrimps (*Fenneropenaeus indicus*) from coastal waters off Cochin, Kerala, India. Their study offers important new information about the dynamics and prevalence of microplastic pollution in the area's marine life. The study highlighted the large contamination levels and seasonal fluctuations by carefully analyzing the presence and distribution of

microplastics in the gastrointestinal tracts of Indian white shrimps. There were found to be 128 microplastics, with fibers making about 83% of the total. The research clarifies the possible effects on marine ecosystems and human health by evaluating the features and abundance of microplastics in these commercially significant crustaceans. The findings also point to the possibility that humans may absorb microplastics through the ingestion of whole dry white shrimp or peeled but unveined shrimp, particularly during the monsoon season.

Adeogun *et al.* (2020) conducted a study to detect and assess the occurrence of microplastics in the stomachs of commercial fish species sourced from a municipal water supply lake in south-western Nigeria. Important information about the amount and distribution of microplastic pollution in the area's aquatic ecosystems is provided by their research. The study investigated the amount of contamination in this freshwater ecosystem by systematically analyzing the presence of microplastics in the gastrointestinal tracts of commercial fish species. According to the research, MPs pose a direct health danger to aquatic creatures in addition to serving as vectors for the spread of infections and environmental pollutants, both established and new. Through evaluation of the prevalence and possible effects of microplastics on fish health and ecosystem integrity, the study highlights the pressing requirement for efficient management approaches to reduce plastic pollution in freshwater environments.

Bordós *et al.* (2019) conducted a study to identify microplastics in fish ponds and natural freshwater environments across the Carpathian basin in Europe. The research offers significant insights into the distribution and existence of microplastic pollution in the regions manmade and natural water environments through thorough examination. By employing advanced identification techniques, the study detected microplastics in these environments, highlighting the pervasive nature of plastic contamination in freshwater ecosystems. In soil samples, polystyrene and polypropylene (PP) were found in greater abundance than polyethylene and polypropylene (PP) in water. Preliminary findings also suggest that fish ponds could serve as MP deposition sites. The results highlight the need for thorough management plans and monitoring programs to reduce microplastic pollution and save aquatic life in the Carpathian Basin.

Nikki *et al.* (2021) investigated the abundance and characteristics of microplastics in commercially important bottom-dwelling finfishes and shellfish from Vembanad Lake, India. Through detailed analysis, the study aimed to assess the prevalence and attributes of microplastic pollution in key species inhabiting this important aquatic ecosystem. The research sheds light on the extent of microplastic contamination in Vembanad Lake, highlighting its potential impacts on

marine life and human health. The findings confirmed the presence of chlorinated Polyvinyl Chloride, polyethylene, polypropylene and polyester in the samples. The study offers important insights into the consequences of microplastic pollution for fisheries and aquaculture in the area by concentrating on commercially important species.

Badola *et al.* (2023) conducted a study on microplastics in the River Ganga and its fishes, focusing on this iconic Himalayan River. The investigation sought to determine whether microplastics were present in the river water and in the fish populations that inhabited there, as well as how far they were distributed. The study offers insightful information about the degree of microplastic pollution in one of the major river systems in India and possible effects on aquatic life. The study advances our knowledge of plastic contamination in Himalayan freshwater ecosystems by investigating the presence of microplastics in River Ganga fish.

Nugnes *et al.* (2022) conducted a study on the toxic impact of polystyrene microplastic particles on freshwater organisms. The research sought to evaluate the detrimental impacts of microplastic pollution on aquatic life in freshwater ecosystems through thorough experimentation. Additionally, the PS-MP's possible genotoxicity and the generation of ROS in C. dubia were identified. When organisms were exposed to PS-MP, changes in their genetic composition and ROS production were observed at concentrations in the order of μg/L, most likely as a result of inflammatory reactions. The study highlights the harmful effects on the survival and health of freshwater animals, offering important insights into the possible ecological ramifications of microplastic exposure. This study adds to our knowledge of the wider ecological effects of plastic pollution in freshwater ecosystems by investigating the harmful effects of microplastic particles made of polystyrene.

Qiang and Cheng (2021) investigated the effects of exposure to polystyrene microplastics on the gonads of zebrafish (*Danio rerio*). The research aimed to evaluate the effects of microplastic pollution on these aquatic species' reproductive health. The study shows the detrimental effects on zebrafish gonadal growth and function, offering important insights into the possible reproductive toxicity of polystyrene microplastics. Male testes showed significantly higher levels of apoptosis, which led to increased expression of p53-mediated apoptotic pathways. Additionally, there was a considerable decrease in the thickness of the testis basement membrane, an indication of histological modification. The study advances our knowledge of the more extensive ecological and physiological effects of microplastic exposure in aquatic ecosystems by analyzing the impairments in gonadal tissues.

Roy *et al.* (2023) investigated the bioaccumulation of polystyrene microplastics and their effects on antioxidant and acetylcholinesterase (AChE) patterns in the freshwater snail *Filopaludina bengalensis* from the Ganges River. The research clarifies the build-up of microplastics in aquatic species and their possible physiological effects by careful investigation. The snails exposed to polystyrene microplastics showed altered antioxidant levels and AChE patterns, which may indicate oxidative stress and neurotoxic consequences. These results demonstrate the harmful effects of microplastic pollution on the health of ecosystems and freshwater species.

Kaloyianni *et al.* (2021) conducted a study to assess the toxicity and functional tissue responses of two freshwater fish species following exposure to polystyrene microplastics. The effects of fish exposure to microplastic on their physiological and biochemical markers were investigated through careful investigation. The research findings indicated detrimental effects on the fish's functional tissues, emphasizing possible disturbances in essential physiological functions. Fish were exposed to PE-MPs, which caused oxidative stress and altered lipid peroxidation, DNA damage, and ubiquitination. The lipid peroxidation and signal transduction pathways that lead to autophagy and apoptosis were also increased. Compared to the bigger PE-MPs, the smaller ones were more effective in causing changes to all of the assessed latter parameters. The knowledge of the ecological effects of microplastic pollution in freshwater ecosystems is enhanced by this research, which looks into the toxicity of polystyrene microplastics.

CHAPTER 3

METHODOLOGY

3.1. QUALITY ASSURANCE

To prevent contamination, glasswares were thoroughly cleaned, sterilized and heated in a hot air oven. Experiments were carried out in a pristine and sterile environment while wearing appropriate laboratory gears. Filter paper and processed slides were handled cautiously to avoid contamination. Samples were consistently covered with aluminium foil and stored at cold temperatures to maintain quality. All the chemicals were prepared in double distilled water. Only assured analytical-grade chemicals were employed for all the preparations and procedures.

3.2. MAINTENANCE OF TILAPIA IN LABORATORY CONDITIONS

The model organism for the present study was the Mozambique Tilapia (*Oreochromis mossambicus*). The classification of it is as follows:

Phylum **:**Chordata
Class **:**Actinopterygii
Order **:**Cichliformes
Family **:**Cichlidae
Genus **:***Oreochromis*
Species **:***O. mossambicus*

After acquiring the permission from the Animal Ethics Committee (GUZ/IAEC/23-24/N20), the fish *O. mossambicus* were collected from ICAR- Central Coastal Agricultural Research institute, Old Goa and Directorate of Fisheries- Freshwater Fish Seed Farm, Keri- Sattari, Goa, India. They were collected and brought to the laboratory. Fishes were acclimated to laboratory conditions for 15 days into 10000 litre capacity tanks previously washed with rock salt and potassium permanganate prior to subjecting them to experiments. The water parameters were maintained at pH (5-10) and temperature at 27ºC to 30ºC. Photoperiod was maintained at 12-16 hours as a day-night cycle. The acclimated healthy and active fishes of uniform size approximately 8-10 cm were selected for experiment.

3.2.1. Experimental set up of lethal concentration (Lc50)

Polystyrene microplastic were obtained from “Quality Polymers, Palghar, Mumbai-Maharashtra, 401208”. Polystyrene beads were characterized using μ-Raman Spectroscopy (Horiba) to confirm the nature of the plastic. The microplastic beads were then sieved through 5 mm pore size sieve. The resulting microplastics were then used for acute toxicity and subsequent toxicity studies. Four glass tanks of size 60 cm length × 30 cm width × 40 cm height, with capacity of 57 L were selected for the experimental setup. Twenty eight fish were selected for the experiment. According to the

OECD Guideline 203 (OECD, 2019), fishes were divided into two groups, one group was considered an experimental group with 7 fish in each tank exposed to Polystyrene microplastic starting with 5 mg/L, 10 mg/L, 15 mg/L, 20 mg/L for 24, 48, 72 and 96 hrs. Another group without microplastic exposure was considered as control. After the 96 hours exposure if 50% deaths were not observed then the fishes were kept for depuration for 4-5 days prior to the second exposure. The exposure is given at regular intervals of different concentrations until 50% of the population dies within 96 hours. Lc50 was calculated using the Probit Analysis Method by Finney (1952).

3.2.2. Experimental set up of Acute Exposure (14 days)

According to the OECD 203 (OECD, 2019). Guidelines, a 14 days exposure setup was made in 4 glass tanks of size 60 cm length× 30 cm width×40 cm height, with capacity of 57 L with 7 fishes in each tank. Four groups were made that included three experimental and one control tanks. Since no LC50 was obtained the following concentrations of PS microplastics were decided for exposure study: 50 mg/L, 250 mg/L and 500 mg/L. During the 14 days exposure period the fishes were observed daily to check their behavioural responses. After the exposure period of 14 days the blood sample was collected from live fish in PBS for genotoxicity assays then fish were sacrificed by euthanizing in ice cold water. Tissue samples (Gill, Gut, and Liver) of *O. mossambicus* were collected and stored at -20°C prior to conducting various biochemical assays.

3.3. ACCUMULATION OF MICROPLASTICS

(Shruti *et al.*, 2022)

Materials required:

Dissection kit, reagent bottles, measuring cylinder, test tubes, stirrer, dropper, micropipettes and tips, test tube stand, beakers, 2.5µg pore size filter paper, etc.

Chemical Preparation:

1. **10% KOH:** Mix 10 g Potassium Hydroxide in 100 ml Distilled Water
2. **Nile red dye:** Add 1mg Nile Red in 1 ml Methanol

Procedure:

Out of 7 sample sizes two fish were dissected for detecting accumulation in 3 major tissues i.e., gill, gut and liver. 5 ml of 10% KOH was added in the tissue and incubated for 24 hours at 60ºC. If the tissue was not digested 3 ml of 30% H_2O_2 was added to the sample and incubated for another 24 hours. In case of very small particle size of MP, Nile Red Dye was added to the mixture (optional) and the extract was then filtered using Whatmann filter paper with a pore size of 2.5µg in a vacuum filtration pump (BORO-017). The filtered samples were then dried at 60ºC for 15

minutes. These filter papers were examined under stereo microscope (Carl Zeiss Microscopy GmbH)/fluorescence microscope (OLYMPUS U-TV0.63×C).

3.4. GENOTOXICITY TESTS

3.4.1. MICRONUCLEUS TEST

(Nagpure *et al.,* 2007)

Materials required:

Reagent bottles, beakers, conical flask, glass slides, whatmann filter paper, blotting paper, measuring cylinder, 0.1 ml syringe, coplin jars, etc.

Chemical Preparation:

1. Giemsa stain:

Add 1 g Giemsa powder in 54 ml Glycerol. Keep the solution on a magnetic stirrer at 56ºC for 2 hours to dissolve the Giemsa powder. Cool the solution and add 84 ml of Methanol. Store the mixture for 24 hours. After 24 hours filter the stain using whatmann filter paper and store the filtered solution as stock at room temperature.

10% working solution: Mix 10 ml Stock solution in 90 ml Phosphate Buffer.

Principle:

The micronucleus assay is a method employed to evaluate the genotoxic and cytotoxic effects on cells. In toxicity studies concerning fish, this assay specifically focuses on analysing blood cells for micronuclei presence. Micronuclei are tiny additional nuclei that arise during cell division when chromosomes fail to segregate correctly. The underlying principle of the micronucleus assay lies in the fact that exposure to genotoxic agents, such as certain chemicals or radiation, can cause DNA damage or disrupt the cell division process, resulting in micronuclei formation. Elevated frequencies of micronuclei compared to control samples indicate potential genotoxic or cytotoxic effects induced by the substances under investigation (Melo *et al.,* 2014).

Procedure:

Blood samples were collected from the live fish at the end of the 14 days exposure. A fresh blood drop (approx. 0.5µl) was placed on a clean slide. With the help of another slide a smear was made. The slides were left to air dry for 5-10 minutes. The slides were fixed by dipping in methanol for 5-6 seconds. After the fixation the slides were air dried again for 5-10 minutes before being immersed in Giemsa stain for 25-30 minutes. After staining, the slides were removed and blot dried. Excess stain was drained by using a Phosphate Buffer. Slides were air dried for 5-10 minutes and fish erythrocytes were observed under Infinix Trinocular Microscope, 19361.

3.4.2. COMET ASSAY

(Nagpure *et al.,* 2007)

Materials required:

Reagent bottles, beakers, conical flask, whatmann filter paper, measuring cylinder, 0.1 ml syringe, coplin jars, eppendorf 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 diydrogen 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) **% Dimethyl sulphoxide (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:**

Mix solution (a) and solution (b) in the stock solution (c)

4. **Electrophoresis 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 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. Make volume up to100 ml and store the solution in 5ºC.

6. **Preparation of Agarose Gel:**

a) **High Melting Agarose:** 0.500 g High Melting Agarose is mixed in a 50 ml Phosphate Buffer. Heat the solution in the 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.

c) **Ethidium Bromide Stain:** For 10x stock solution add 10 mg Ethidium Bromide in 50 ml Double Distilled Water. Stir the solution and store in an amber bottle in the dark. For 1x working solution add 1 ml stock solution in 9 ml Double Distilled Water.

Principle:

The Comet assay offers delicate means of evaluating DNA damage at the level of individual cells. It involves examining electrophoretic movement of DNA fragments derived from nucleotides post cell lysis within the thin layer of agarose. If the DNA is damaged the broken Strands will appear like a Comet Tail under the fluorescence light (Afanasieva and Sivolob, 2018).

Procedure:

The blood sample was collected in the eppendorf tube containing PBS. Clean and dry frosted glass slides were taken for the assay. On the slides a thin layer on High Melting Agarose was made. Then the slides were kept for drying till the agarose was dried and set on the slide. After the first layer, the second layer was made by mixing equal volumes of sample and Low Melting Agarose and the layer was allowed to set for 5-10 minutes. The third layer was made using only Low Melting Agarose and slides were allowed to dry completely. All the prepared slides were kept in Lysis Buffer for 24 hours. After 24 hours, the slides were introduced to the Electrophoresis buffer and the electrophoresis unit was activated. Following electrophoresis, the slides were dried by blotting 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 allowed to set for 15 minutes. Then the slides were observed under fluorescence microscope (OLYMPUS U-TV0.63×C).

3.5. HISTOLOGY

Materials required:

Dissection kit, glass vials, tissue, dropper, etc.

Chemicals Required:

1. 10% Formalin
2. 70% Ethanol

Histology investigates the morphology, organization, specialized functions and extracellular matrix components of cells within tissues, analysing their structure and function. It establishes a fundamental comprehension of normal tissue structure and function, serving as a standard for assessing toxicological conditions. This involves identifying cellular abnormalities, inflammation, necrosis, fibrosis and disturbances in cellular or tissue integrity (Ross *et al.,* 2010).

Gills and gut for histological examination were washed thoroughly with physiological saline, weighed and stored in 10% Formalin for 24 hours to arrest the present stage of the tissue. After that the tissue was kept in 70% Ethanol for dehydration, then the samples were embedded in paraffin wax and 6 micron thick sections were prepared on the slide. The slides were then stained with hematoxylin stain and eosin stain. The prepared slides were observed under a Trinocular Microscope (Infinix 9361)

3.6. PREPARATION OF SAMPLES FOR BIOCHEMICAL ASSAY

The gill, gut and liver organs of the fishes were dissected, kept in ice cold buffered saline (pH 7.2) and homogenized using a tissue homogenizer (Remi L). The obtained suspension was centrifuged at 4500 rpm for 15 min in cold centrifuge EMIR-24. This supernatant is then used for biochemical enzyme analysis. For all the colorimetric biochemical tests VIS spectrophotometer LMSP-V325 was used.

3.7. BIOCHEMICAL ASSAY

A biochemical assay refers to an analytical method used to identify and measure cellular processes such as enzymatic reactions, metabolic reactions and cell signalling. These assays serve as dependable and frequently employed techniques for characterising targets and gaining insights into biomolecular functions. The biochemical enzyme examination of antioxidant parameters (CAT, TBARS), macromolecules (Protein, Carbohydrate) liver function tests (ALT, ALP) were performed on stored supernatant of gill, gut and liver tissues of both control and exposed groups.

3.7.1. CATALASE ASSAY

(Aebi, 1974)

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 (H_2O_2):**

Mix 1.88 ml H_2O_2 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.

Principle:

The catalase activity assay measures the rate at which catalase enzymes catalyse the decomposition of H_2O_2 into H_2O and O_2 This is typically done by monitoring the decrease in absorbance of H_2O_2 at specific wavelengths. The rate of oxygen evolution in catalase assay is measured directly monitoring the decrease in H_2O_2 concentration by spectrophotometrically as H_2O_2 decomposes absorbance decreases (Beers *et al.*, 1951).

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.

3.7.2. THIOBARBITURIC ACID REACTIVE SUBSTANCES (TBARS):

(Niehaus *et al.*, 1968)

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. **TBA-TCA-HCL Reagent: (1:1:1)**

a) **Thiobarbituric acid:**

0.185g of Thiobarbituric acid powder was added to 50 ml of Double distilled water.

b) **Trichloroacetic acid**

Add 7.5g Trichloroacetic acid in 50 ml Double distilled water.

c) **0.25N HCL**

1. O44ml of concentrated HCL is added to 48.95ml of Double distilled water.

Mix solution (a), (b), and (c) in 1:1:1 volume

Principle:

The principle behind the TBARS assay is measuring lipid peroxidase product, with a focus on Malondialdehyde (MDA) to gauge oxidative stress. This method relies on interaction between MDA and TBA in acidic environments resulting in the formation of a pink-red coloured complex that can be measured using spectrophotometry. The intensity of the colour corresponds to the concentration of MDA, enabling the evaluation of oxidative damage in biological samples (De Leon *et al.,* 2020)

Procedure:

Freshly obtained tissues were homogenized in 5 ml Tris-HCl Buffer. The sample was treated with 2 ml of TBA-TCA-HCl reagent and placed in a boiling water bath for 15 minutes. Cooled and centrifuged at room temperature for 10 minutes at 5000 rpm**.** The absorbance of clear supernatant was observed against a suitable blank at 535 nm.

3.7.3. ALANINE TRANSAMINASE ASSAY (ALT):

(Reitman and Frankel, 1957)

Materials required:

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

Chemical preparation:

1. **Standard Pyruvate:**

Add 0.22 g 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 weighted compounds and make the volume up to 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. **DNP Hydrazine (DNPN):**
   Weigh 0.05 g of DNP and mix with 46.4 ml Concentrated HCl. Adjust the volume to 100 ml using Distilled Water.

Principle:

The ALT assay, also known as Alanine Transaminase assay, is a diagnostic procedure utilized for assessing ALT enzyme levels in the blood or tissue. Its principle involves the conversion of alanine to pyruvate facilitated by ALT enzymes with the simultaneous conversion of α-ketoglutarate to glutamate. The enzymatic reaction generates an equimolar quantity of NADH, which can be quantified via spectrophotometry at a specific wavelength. The elevated ALT levels may indicate tissue damage or disease (Hsueh *et al.*, 2011).

Procedure:

Five 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 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°C 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.

3.7.4. ALKALINE PHOSPHATASE (ALP):

(King and Armstrong, 1934)

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 up to 50 ml using Distilled water.
2. **ALP Standards:**
   Mix 5 mg of p-nitrophenol in 5 ml of Distilled water
3. **0.025 N Sodium Hydroxide (NaOH):**
   Mix 0.04 g of Sodium Hydroxide in 40 ml of Distilled water

4. **ALP Substrate:**

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

Principle:

Alkaline Phosphatase Assay is used to gauge the extent of stress or damage induced by exposure to toxins. This stress response often triggers an elevation in ALP activity. The ALP assay test assesses the activity of Alkaline Phosphatase enzyme within the sample that involves hydrolysis of a colourless substrate by ALP enzyme, resulting in the generation of pale yellow-coloured product (Adams *et al.,* 1997).

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 introduced in the sample mixtures and the OD was measured at 405 nm.

3.7.5. PROTEIN ESTIMATION

(Lowry *et al.,* 1951)

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 Tartrate:

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

c) 2% CuSO4:

Add 0.1 g CuSO4 in 5 ml Distilled water.

Mix 147 ml of solution (a), 1.5 of solution (b) and 1.5 ml CuSO4 to prepare Lowry's reagent.

3. Folin's Reagent:

Mix 5 ml of Folin's reagent in 10 ml Distilled water.

Principle:

Lowry's method for protein estimation operates on the principle of proteins interacting with copper ions and Folin-Ciocalteu reagent. When proteins are present in the sample, copper ions undergo reduction to cuprous ions, which then react with the Folin-Ciocalteu reagent under alkaline conditions, leading to the formation of a blue-coloured complex. Moreover, the presence of tyrosine and tryptophan residues within proteins triggers the reduction of phosphomolybdate and phosphotungstate components of Folin-Ciocalteu reagent, resulting in formation of bluish products. This contributes in enhancing the sensitivity of the method, aiding in its accuracy for protein quantification. The intensity of the blue colour is directly proportional to the protein concentration in the sample and can be quantified using spectrophotometry at a specific wavelength (Shen, 2023).

Procedure:

a) Extraction:

For the extraction of protein the freshly prepared homogenate was treated with 7 ml of 10% Perchloric acid and centrifuged at 4000 rpm for 10 minutes. The supernatant was discarded and 4 ml Chloroform and 2 ml Methanol added to the residue. The sample was centrifuged again at 4000 rpm for 10 minutes. The residue was collected after centrifuging and treated with 1.5 ml 10% Perchloric acid. Samples were centrifuged at 4000 rpm for 10 minutes. After that the residue was collected and 2 ml of 1N NaOH was added to the samples and kept in a boiling water bath for dissolving the residues completely. The clear solution obtained was 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 s, freshly prepared homogenate supernatant sample was taken. Both the standard 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 10 minutes. After the appearance of blue coloration the OD was measured at 660 nm using a spectrophotometer.

3.7.6. CARBOHYDRATE ESTIMATION

(Hedge and Hofreiter, 1962)

Materials required:

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

Chemical preparation:

1. Standard Glucose Solution

Add 10 mg Glucose in 100 ml Distilled water.

2. Anthrone reagent:

Weigh 0.4 g Anthrone Powder and mix with 200 ml Concentrated Sulphuric Acid

Principle:

The Anthrone reagent functions as a calorimetric indicator for carbohydrate estimation. Under the acidic conditions and upon heating with the anthrone reagent, carbohydrates form a complex that displays a blue-green hue. The intensity of this colour directly correlates with the concentration of carbohydrates in the sample that is predetermined at specific wavelength using a spectrophotometer (Sapkota, 2022).

Procedure:

1 ml of supernatant from centrifuged tissue homogenate was taken. Carbohydrate stock solution of Glucose was taken as a standard sample. Both samples were treated with 4 ml Anthrone reagent and kept for incubation in a hot water bath for 15 minutes. The blue green colouration indicated the presence of carbohydrates. Then the OD was taken at 620 nm.

3.8. STATISTICAL ANALYSIS

Statistical Analysis was done by using Graphpad Prism 10 Software. The datasets were characterized by presenting the mean values accompanied by standard deviations to discern significant differences in both toxicological parameters and trace microplastics. One-way analysis of variance (ANOVA) was employed for this purpose. To further analyze statistically significant differences between two groups, Dunnett's post hoc test was applied. Additionally, correlation analysis was conducted to explore the relationship between toxicological indicators. Statistical significance was determined at $p<0.05$, $p<0.01$ and $p<0.001$ levels.

CHAPTER 4
ANALYSIS AND CONCLUSION

4.1. RESULTS

4.1.1. CHARACTERIZATION OF POLYSTYRENE (PS) MICROPLASTIC

µ-Raman spectrometer was used to validate the identity of the polystyrene microplastic. The generated wavelength spectrum was compared to the polystyrene microplastic reference spectrum and was validated.

4.1.2. Lc50

The acute toxicity exposure was carried out to determine the lethal concentration (Lc50) of Polystyrene (PS) microplastics on *Oreochromis mossambicus.* The exposure to different concentrations of PS microplastics resulted in no significant lethality even after 96 hours of exposure. Hence, it can be concluded that tilapia (*O. mossambicus*) exhibit significant resistance to Polystyrene microplastic and demonstrate a low mortality rate for Polystyrene microplastic in acute exposure.

4.1.3. BEHAVIOURAL OBSERVATIONS:

Throughout the 14-day exposure of PS microplastics to *O. mossambicus*, the fish's behaviours were observed closely. Upon initial exposure, some fish displayed feeding behaviour, but after realizing that the particles were not food, they expelled the microplastic particles from their mouths. During this process, the fish inevitably ingested some of the microplastic particles. Additionally, a few fish exhibited hyperactive behaviours such as fast swimming, chasing and nipping other fish. While others became notably solitary at the corners of the tank to avoid the microplastic particles. Additionally, the rate of gasping increased proportionally to the dosage in all treatment group fish.

4.1.4. ACCUMULATION OF PS MICROPLASTIC

This test involves investigating the accumulation and distribution of PS microplastics in different tissues (gill, gut, and liver). The findings of the study confirmed that exposure to the PS microplastics lead to their increased accumulation in specific tissues in a concentration-dependent manner. Specifically the accumulation was observed higher in gill, followed by gut and liver. Accumulation of PS microplastic in gill, gut, and liver is shown in (Plate V and VI).

4.1.5. BIOCHEMICAL ASSAY

a) Catalase assay

The results for catalase estimation of *O. mossambicus* exposed to polystyrene microplastics are shown in graph 4.1. The catalase concentration was found to increase with increasing microplastic concentrations.

Significant variation in gill Catalase activity was observed across all the groups ($F=11.16$; $p<0.01$). The results for gill tissue showed that the catalase concentration was highest in experimental group **3.** Dunnet's multiple comparison tests showed significant differences between experimental 2 and experimental 3 compared to the control. The results of the assessment of catalase in the gut indicated that experimental 3 had the highest values. The catalase activity in the gut tissues was found to have a p value of $p<0.001$ and an F value of 12.62 according to the ANOVA results. Dunnet's multiple comparison tests showed significant difference in control with experimental 3. In the liver tissue the highest level of enzyme activity was seen in experimental group 3 compared to control group, with ANOVA results indicating $p<0.01$** and $F=6.942$. Significant differences between control and experimental group 3 were revealed by Dunnet's multiple comparison tests.

b) TBARS Assay

The results of the Thiobarbituric acid reactive substances estimation for *O. mossambicus* exposed to PS microplastics are shown in Graph 4.2. It was discovered that the concentration of Malondialdehyde increased with an increase in microplastics concentration.

The results for the gill tissue indicated that experimental 3 had the greatest MDA concentration, with an ANOVA result of $F=21.35$ and $p<0.0001$. There was a significant difference in the experimental group 2 and experimental group 3 compared to the control group, according to Dunnet's multiple comparison tests.

The gut's MDA levels were analyzed to be the highest in experimental group 3. The MDA activity in the gut tissues had a p value of $p<0.01$ and an F value of 6.234, according to the ANOVA results. Dunnet's multiple comparison tests showed a correlation between the experimental 2 and experimental 3 groups with the control group. In comparison to the control, and experimental groups, the ANOVA findings indicated that experimental group 3 had the highest level of enzyme activity in the liver tissue ($p<0.001$ and $F=12.78$). The results of Dunnet's multiple comparison tests indicated a highly significant difference between the control and experimental group 3.

c) ALT Test

The results of the Alanine transaminase estimation for *O. mossambicus* exposed to PS microplastics are shown in Graph 4.3. It was discovered that the ALT concentration increased with an increase in microplastics concentration.

The results for the gill tissue indicated that experimental group 3 had the greatest ALT concentration, with an ANOVA result of $F=6.426$ and $p<0.01$. There was a significant difference in the experimental group 3 compared to the control group, according to Dunnet's multiple comparison tests. The gut's ALT levels were analyzed to be the highest in experimental group 3. The ALT activity in the gut tissues had a p value of $p<0.0001$ and an F value of 25.42, according to the ANOVA results. Dunnet's multiple comparison tests showed a strong correlation between the experimental and control groups. In comparison to the control, experiment 2, and experiment 3 groups, the ANOVA findings indicated that experimental group 3 had the highest level of enzyme activity in the liver tissue ($p<0.01$ and $F=6.461$). The results of Dunnet's multiple comparison tests indicated a highly significant difference between the control and experimental group 3.

d) ALP Test

Graph 4.4 displays the Alkaline Phosphatase estimation findings for *O. mossambicus* exposed to PS microplastics. It was found that when the concentration of microplastics increased, correspondingly the ALP concentration also increased.

The results for gill tissue showed that the catalase concentration was highest in experimental group 3, showing the ANOVA results as $F=15.04$; $p<0.0001$. Dunnet’s multiple comparison tests showed significant difference in control with respect to all the experimental groups.

According to the analysis of the gut's ALP levels, experimental group 3 had the highest values. The ANOVA results showed that the ALP activity in the gut tissues had a p value of $p<0.001$ and an F value of 9.423. Dunnet's multiple comparison tests revealed a noteworthy distinction between control and experimental group 3.

ANOVA results showed that experimental group 3 had the highest level of enzyme activity in the liver tissue when compared to the control, experiment 2, and experiment 3 groups ($p<0.001$ and $F=12.25$). Dunnet's multiple comparison tests showed significant differences between experimental group 3 and control.

e) Protein Estimation

Graph 4.5 shows the protein estimate results for the gill, gut, and liver tissues in the control and PS microplastics treated groups. The one-way ANOVA test revealed that there was no significant change in the protein concentration of the gut and gill tissue between the control and experimental groups. A one-way ANOVA revealed that the liver tissue obtained had a p value of $p<0.05$ and $F=4.202$, indicating significance. For the same liver tissue, the Dunnet's test revealed a significant difference between control and experimental group 3.

f) Carbohydrate Estimation

As the concentration of microplastics increased, it was found that the content of carbohydrates reduced. Graph 4.6 displays the results of carbohydrate estimation for the liver, gut, and gill tissues of *O. mossambicus* subjected to PS microplastics. The tissues of the control group had the highest concentration of carbohydrates, whereas the tissues of the experimental groups had decreasing concentrations of carbohydrates, with group 3 having the lowest concentration. A one-way ANOVA revealed a significant variation in the gut tissues, with a p value of $p<0.5$ and an $F=4.716$. The results of the multiple comparisons Dunnet's test indicated a significant difference between experimental group 3 and control. The liver and gill tissues showed no noticeable variations.

4.1.6. PEARSON'S CORRELATION

For PS microplastics exposed *O. mossambicus* fishes, Pearson's correlation was applied for 3 different tissues of 3 exposed groups and control fishes for gill, gut and liver tissue. The correlation was analysed for six different tests (Catalase assay, ALP Test, ALT Test, TBARS Assay, Protein and Carbohydrate Estimation).

Pearson's correlation result for gills presented in table 4.1, showed positive correlation between ALP-Catalase ($p<0.05$); ALT-Catalase ($p<0.05$); ALT-ALP ($p<0.0001$) and TBARS-Catalase ($p<0.05$); TBARS-ALP ($p<0.001$); TBARS-ALT ($p<0.01$) and negative correlation between Protein-ALP ($p<0.05$).

Pearson's Correlation for the Gut tissue is shown in table 4.2. The results indicate positive correlation between ALP-Catalase ($p<0.0001$); ALT-Catalase ($p<0.001$); ALT-ALP ($p<0.01$) and negative correlation between TBARS-Catalase ($p<0.01$); TBARS-ALT ($p<0.01$); Protein-Catalase ($p<0.05$); Protein-ALP ($p<0.05$); Protein-ALT ($p<0.01$) and negative correlation between Carbohydrate-ALP ($p<0.05$).

For liver tissue Pearson's Correlation results are presented in table 4.3 indicating positive correlation between ALP-Catalase ($p<0.0001$); ALT-ALP ($p<0.05$); TBARS-Catalase ($p<0.0001$); TBARS-ALP ($p<0.0001$); TBARS-ALT ($p<0.05$); Carbohydrate –ALT ($p<0.05$).
Maximum correlation was observed in gut tissue followed by gill tissue and least in liver tissue.

4.1.7. HISTOLOGY

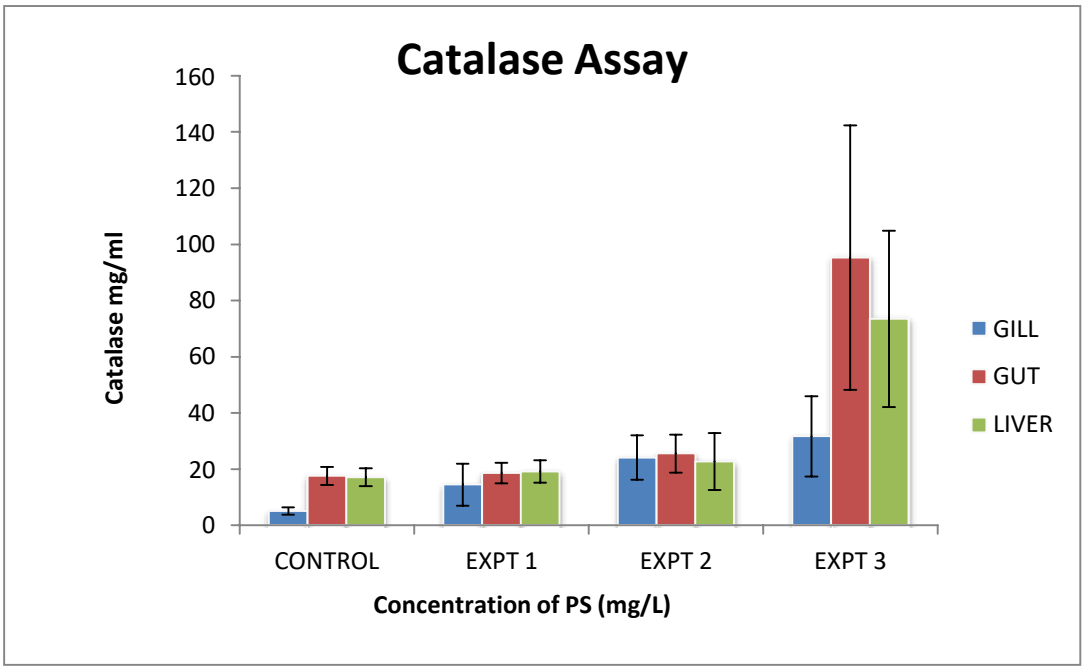
The resulting slides of histological analysis for gill and gut tissue of control and PS microplastics exposed *O. mossambicus* are represented in plate IX and X respectively.
For the gill tissue from the control group, normal structures of the gill were observed having intact primary lamellae, secondary lamellae, cartilaginous matrix and erythrocytes. A prominent difference was observed for microplastic exposed gill tissues wherein disruption of the overall tissue was seen with disrupted lamellae structures along with displaced cartilaginous matrix with decreased number in erythrocytes.
Histology of gut tissue from the control group was observed to be having normal structure with intact serosa and muscularis membrane and intact villi structures. Experimental group tissue samples indicated damaged structures due to PS microplastic exposure resulting in damaged villi and distortion of epithelial lining.

4.1.8. GENOTOXICITY ASSAYS

In the assessment of genotoxicity effects of Polystyrene microplastics in *O. mossambicus*, the micronucleus test and comet assay are commonly utilized to detect DNA damage. In the present study no significant difference was observed in the comet assay between the control and experimental groups. The lack of significant variance between control and experimental samples indicates that the analyzed exposed fish did not display DNA strand breaks.
The results of micronuclei are presented in graph 4.5. The presence of micronuclei in erythrocytes of exposed *O. mossambicus* is shown in plate VIII. The findings of the present study indicate that the clastogenic effects, as evidenced by micronucleus formations, generally increased with increased microplastics concentration i.e., Control<Experimental 1<Experimental 2<Experimental 3. One-way ANOVA showed significant variation in the micronuclei found in cells exposed to different concentrations of PS microplastics, since F and p values were $F=343.1$ and $p<0.0001$.

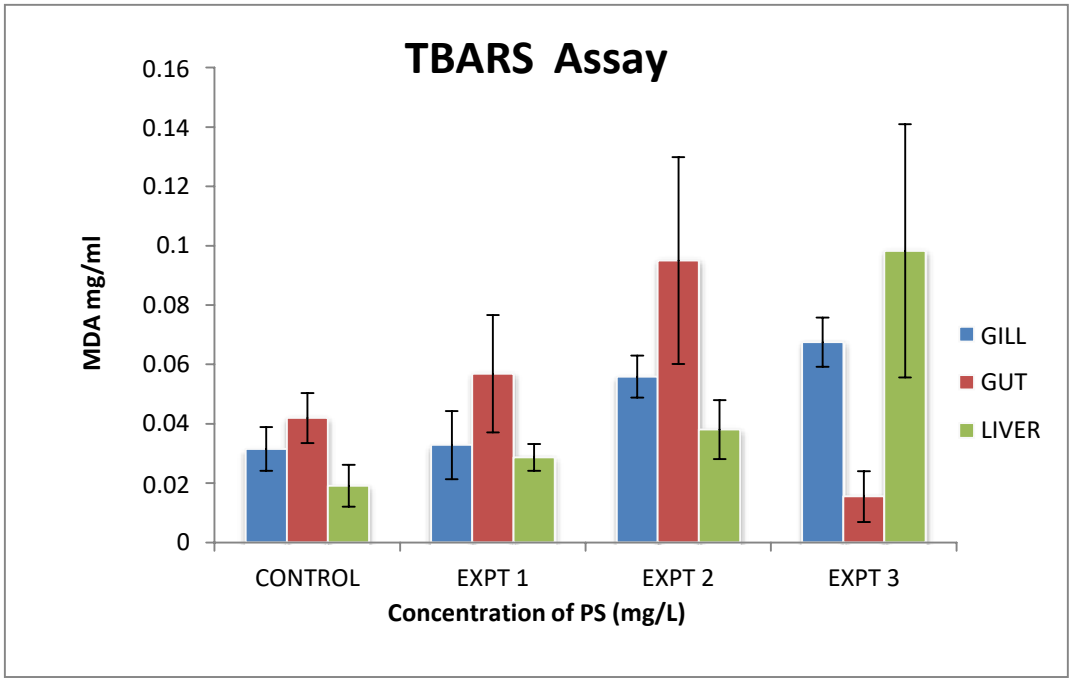
GRAPHS FOR ENZYMATIC ANALYSIS

Graph 4.1 Estimation of Catalase in gill, gut and liver tissues of *O. mossambicus*



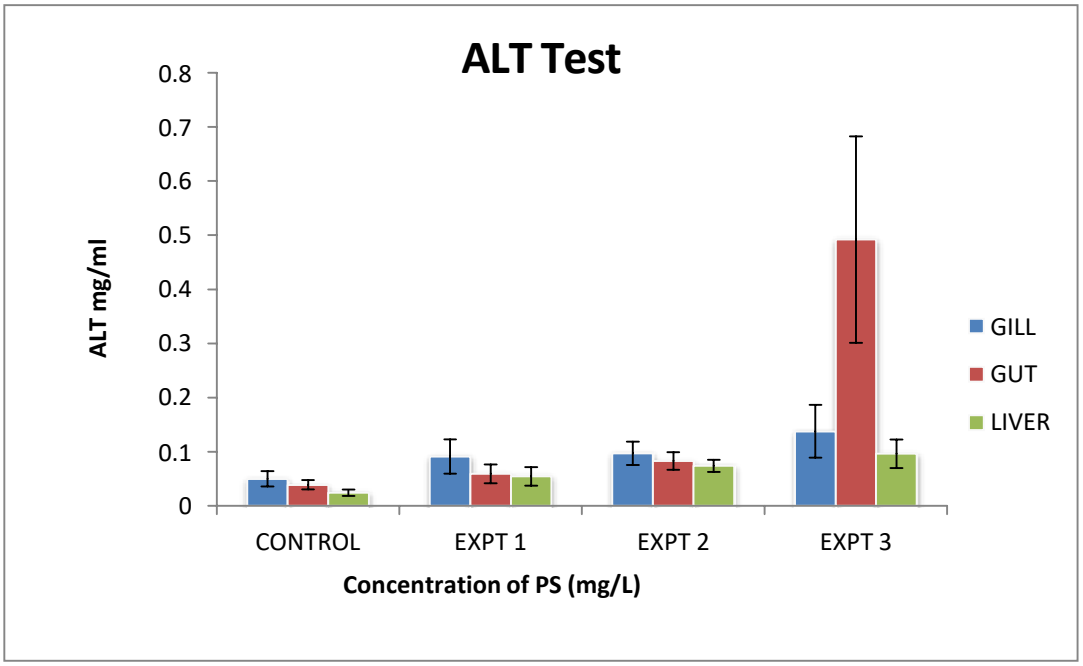
Gill- (F=11.16; p<0.01); **Gut**- (F=12.62; p<0.001); **Liver**- (F=6.942; p<0.01)

Graph 4.2 Estimation of Thiobarbituric acid reactive substances in gill, gut and liver tissues of *O. mossambicus*



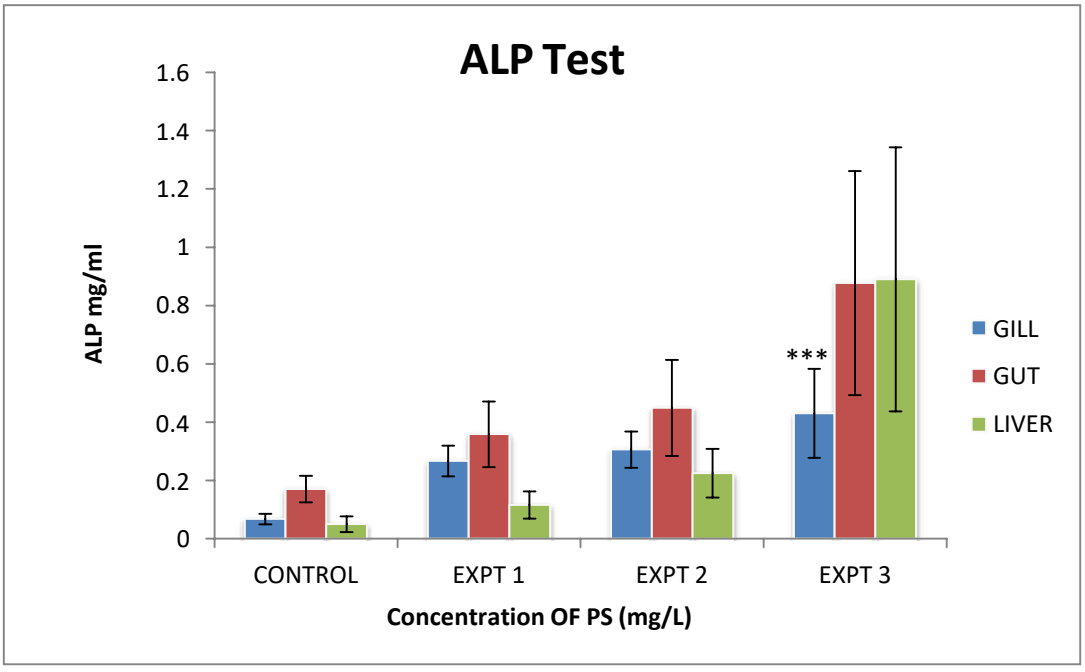
Gill- (F=21.35; p<0.001); **Gut**- (F=6.234; p<0.01); **Liver**- (F=12.78; p<0.001)

Graph 4.3 Estimation of Alanine transaminase in gill, gut and liver tissues of *O. mossambicus*



Gill- (F=6.426; *p*<0.01); **Gut**- (F=25.42; *p*<0.001); **Liver**- (F=6.461; *p*<0.01)

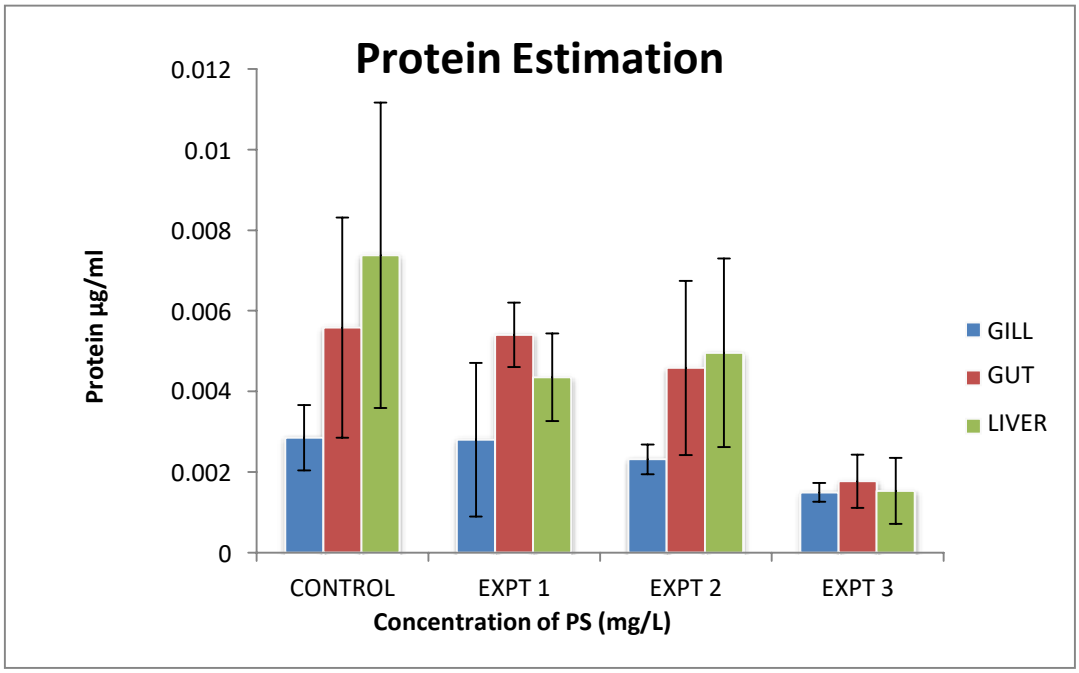
Graph 4.4 Estimation of Alkaline phosphatase in gill, gut and liver tissues of *O. mossambicus*



Gill- (F=15.04; *p*<0.0001); **Gut**- (F=9.423; *p*<0.001); **Liver**- (F=12.25; *p*<0.001)

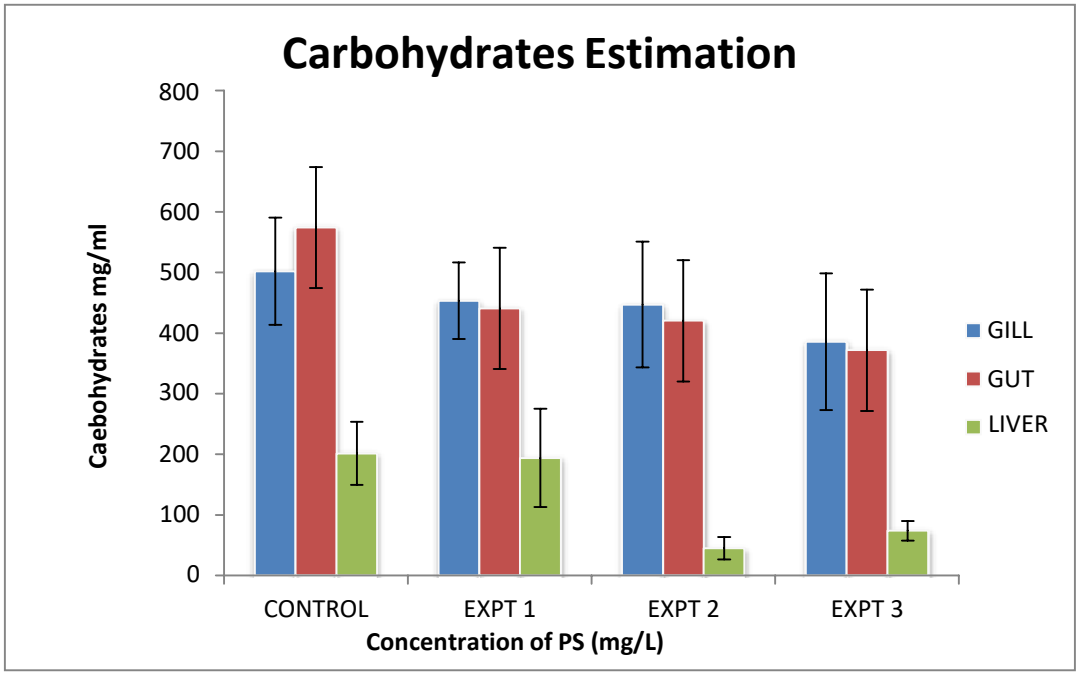
GRAPHS FOR NUTRIENT PROFILING

Graph 4.5 Estimation of Protein in gill, gut and liver tissues of *O. mossambicus*



Gill- (F=1.289; *p*=ns); **Gut**- (F=2.936; *p*=ns); **Liver**- (F=4.202; *p*<0.05)

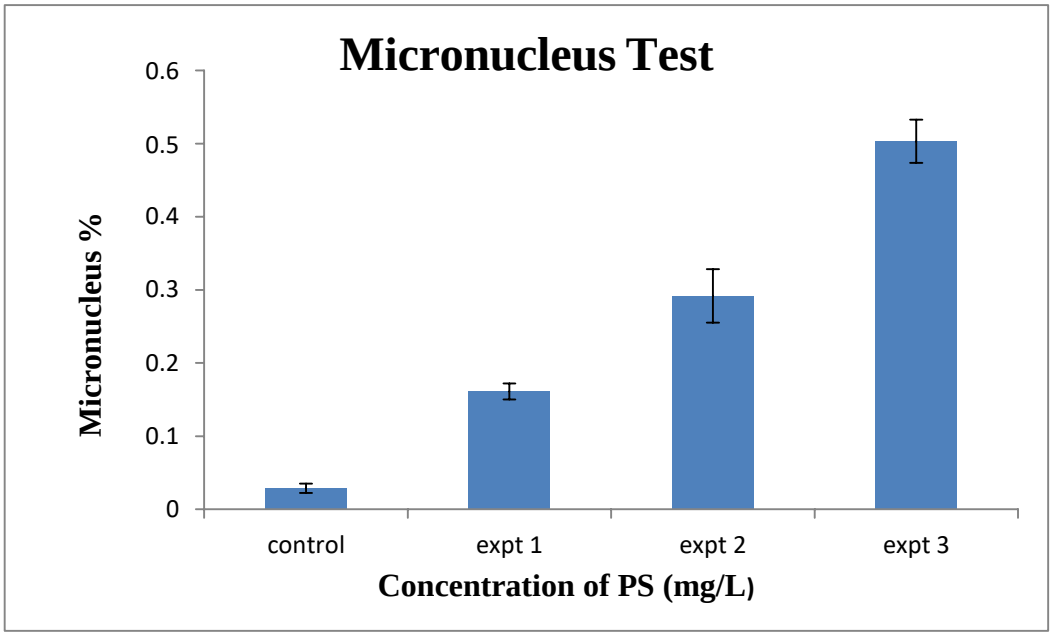
Graph 4.6 Estimation of Carbohydrtes in gill, gut and liver tissues of *O. mossambicus*



Gill- (F=1.988; *p*=ns); **Gut**- (F=4.716; *p*<0.05); **Liver**- (F=1.243; *p*=ns)

GRAPH FOR GENOTOXICITY TEST

Graph 4.7 Micronucleus test for *O. mossambicus* exposed to different concentrations of PS microplastics



F=343.1; $p<0.0001$***

Table 4.1 Pearson's correlation of gill tissue of *O. mossambicus* exposed to different concentrations of PS microplastics

	CATALASE	ALP	ALT	TBARS	PROTEIN	CABOHYADRATES
CATALASE	-					
ALP	**0.5534***	-				
ALT	**0.5222***	**0.8728*****	-			
TBARS	**0.6542***	**0.7213*****	**0.6028****	-		
PROTEIN	-0.4123	**-0.4777***	-0.2848	-0.4173	-	
CARBOHYDRATES	-0.3164	-0.1615	-0.2428	-0.1557	-0.0367	-

Table 4.2 Pearson's correlation of gut tissue *O. mossambicus* exposed to different concentrations of PS microplastics

	CATALASE	ALP	ALT	TBARS	PROTEIN	CABOHYADRATES
CATALASE	-					
ALP	**0.8835*****	-				
ALT	**0.7080*****	**0.6353****	-			
TBARS	**-0.5779****	-0.3506	**-0.5686****	-		
PROTEIN	**-0.4932***	**-0.5208***	**-0.6099****	0.2144	-	
CARBOHYDRATES	-0.2783	-0.3653	**-0.5683****	0.1866	0.2396	-

Table 4.3 Pearson's correlation of liver tissue *O. mossambicus* exposed to different concentrations of PS microplastics

	CATALASE	ALP	ALT	TBARS	PROTEIN	CABOHYADRATES
CATALASE	-					
ALP	**0.9742*****	-				
ALT	0.3438	**0.5219***	-			
TBARS	**0.8242*****	**0.8487*****	**0.4736***	-		
PROTEIN	-0.3151	-0.3429	-0.3164	-0.3297	-	
CARBOHYDRATES	-0.3137	-0.3933	**-0.5323***	-0.3527	0.1810	-

PLATE I: Aquiring and maintenance of Tilapia under laboratory conditions

Figure 1 ICAR-Central Coastal Agricultural Research Institute-Old Goa, Goa

Figure 2 Directorate of Fisheries, Fish Seed Farm, Sanquelim, Keri, Goa

Figure 3 Acclimatization of *O. mossambicus* in cemented tank (10000 L) in animal house

PLATE II: Acquiring the Microplastics

Figure 4 Polystyrene microplastics acquired from Quality Polymers

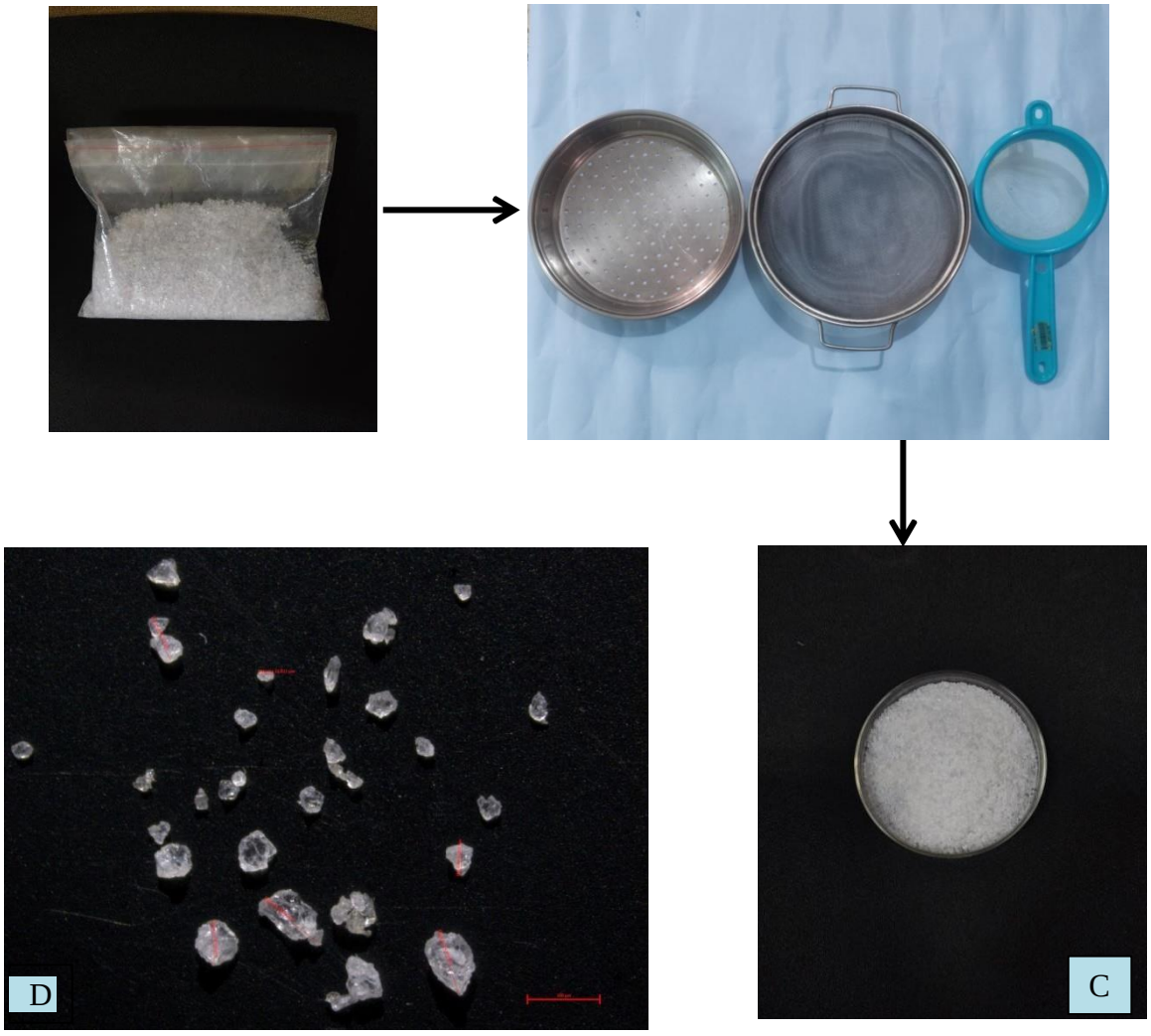


Figure 5 (A) Raw Polystyrene microplastic; (B) Seiving setup-Pore size-0.5 mm; (C) Seived microplastic for exposure; (D) Polystyrene microplastic under Stereo Microscope

PLATE III: Characterization of PS Microplastic

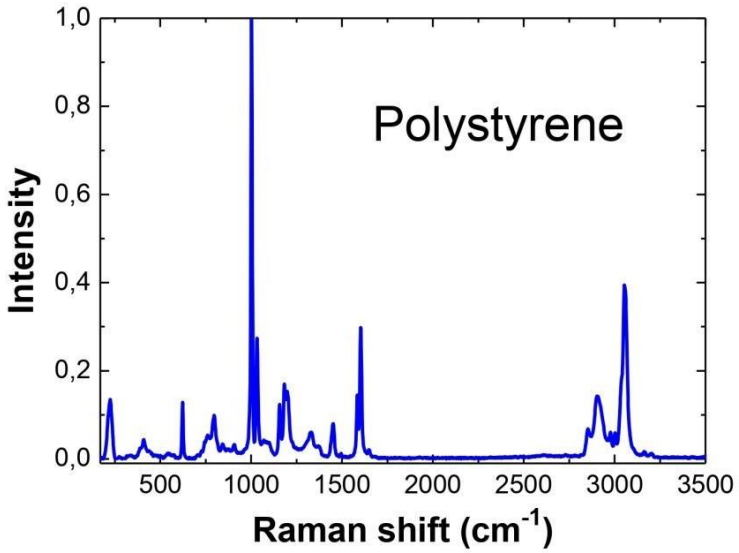


Figure 6 Standard Raman Spectrum of Polystyrene

Source: https://ramanlife.com/library/polystyrene-raman/

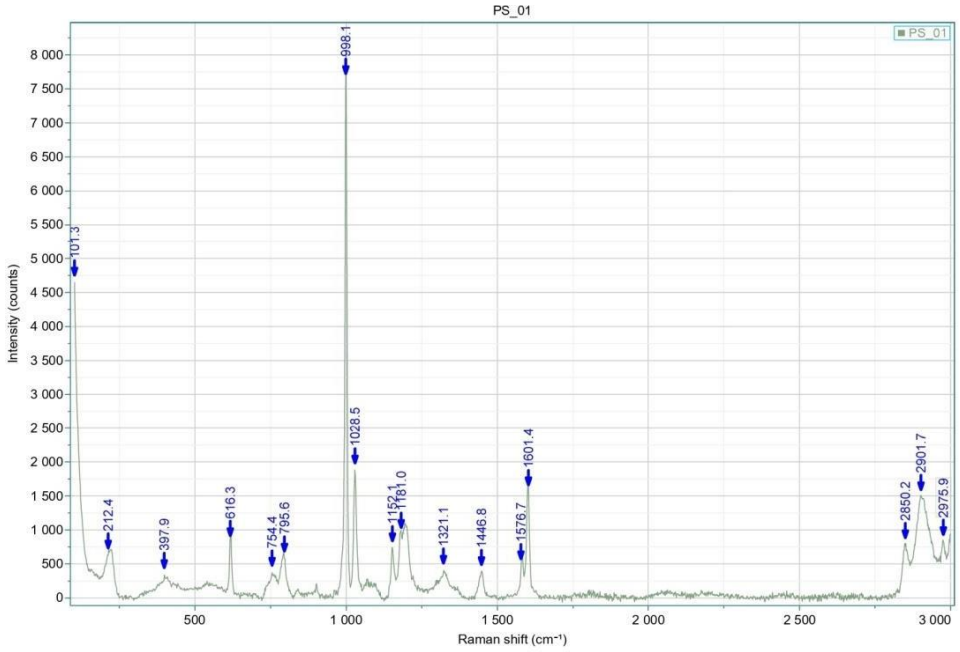


Figure 7 Raman Spectrum of Experimental Polystyrene

PLATE IV: Experimental Setup (Lc50 & 14 days Exposure)

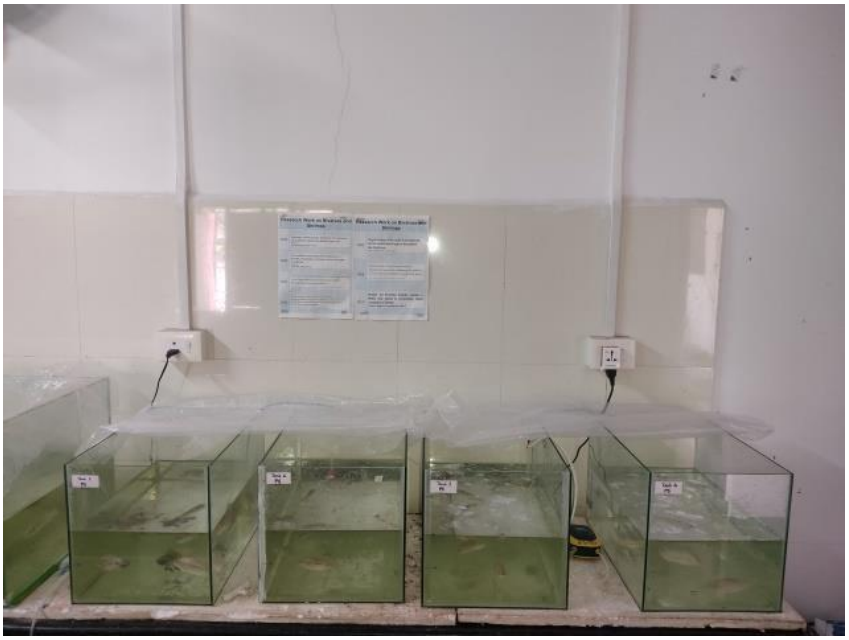


Figure 8 **Exposure Setup**

Figure 9 **Model organism-Oreochromis mossambicus**

PLATE V: Detection of Microplastic in *O. mossambicus*

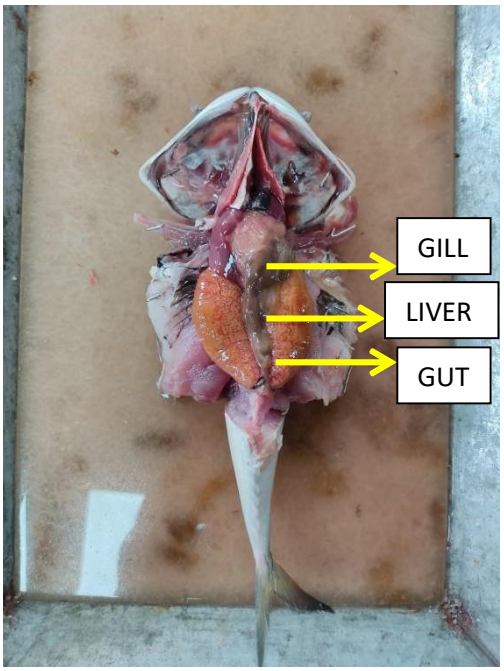


Figure 10 Dissected fish

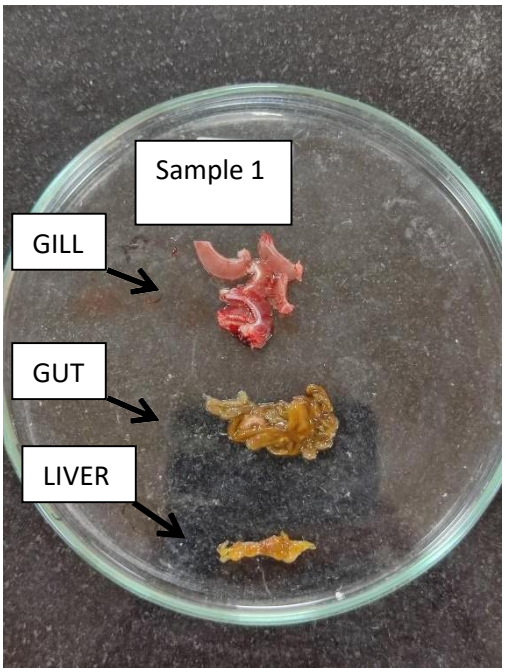


Figure 11 Dissected fish tissue samples

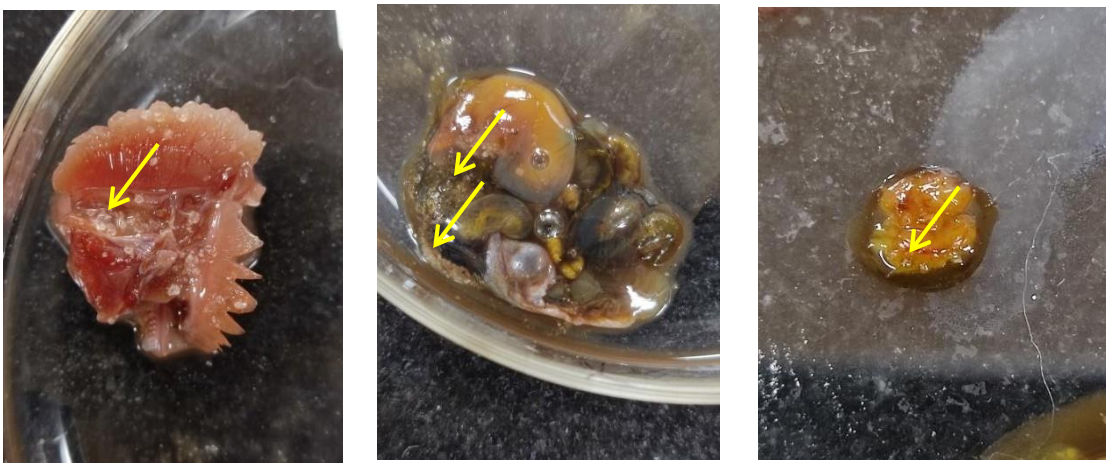


Figure 12 Detection of microplastic in different tissues of *O. mossambicus*; (A) Gill; (B) Gut; (C) Liver

Arrow pointing at microplastic particles present in the samples

PLATE VI: Accumulation of microplastics in *O. mossambicus* after 14 days exposure

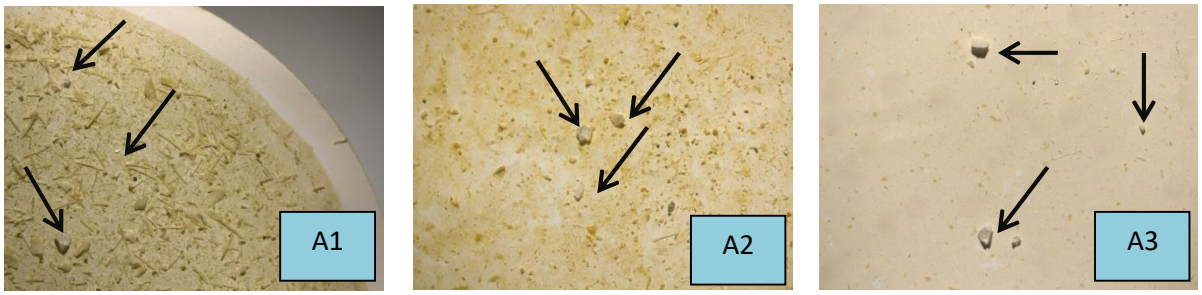


Figure 13 Experimental 1 Group with microplastics (A1) Gill; (A2) Gut; (A3) Liver

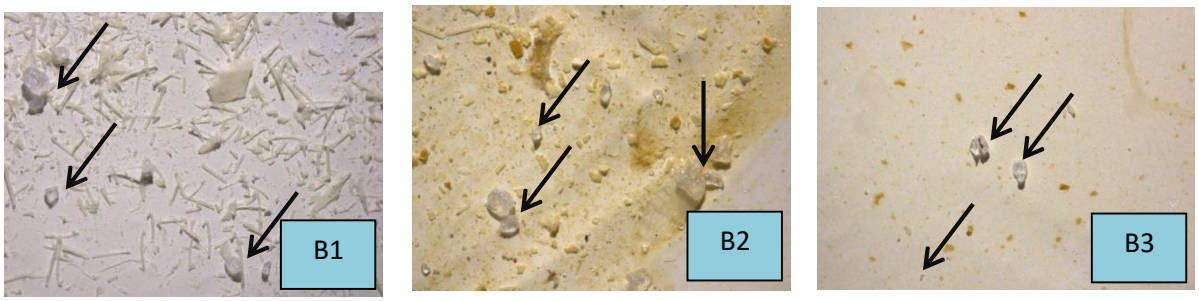


Figure 14 Experimental 2 Group with microplastics (B1) Gill; (B2) Gut; (B3) Liver

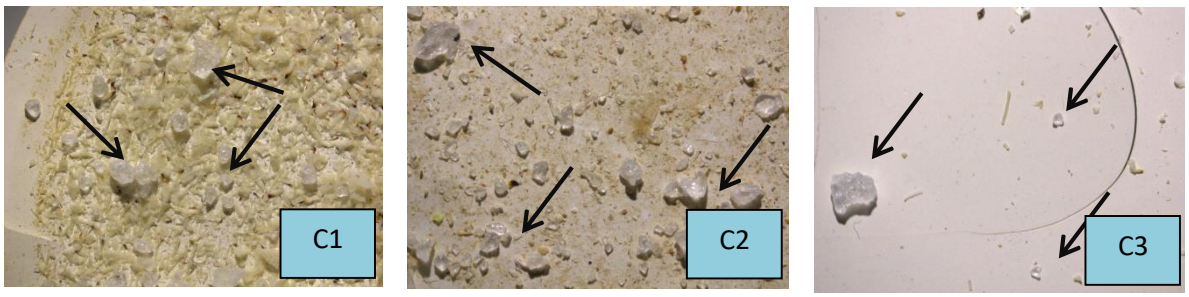


Figure 15 Experimental 3 Group with microplastic-(C1) Gill; (C2) Gut; (C3) Liver

Arrow pointing at microplastic particles present in the samples

PLATE VII: Micronucleus Test

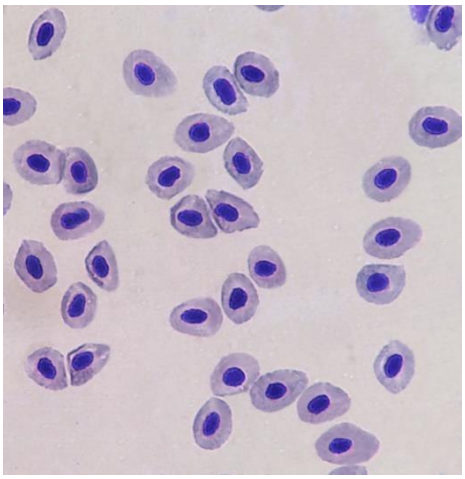


Figure 16 **Erythrocytes of *O. mossambicus* indicating normal cells**

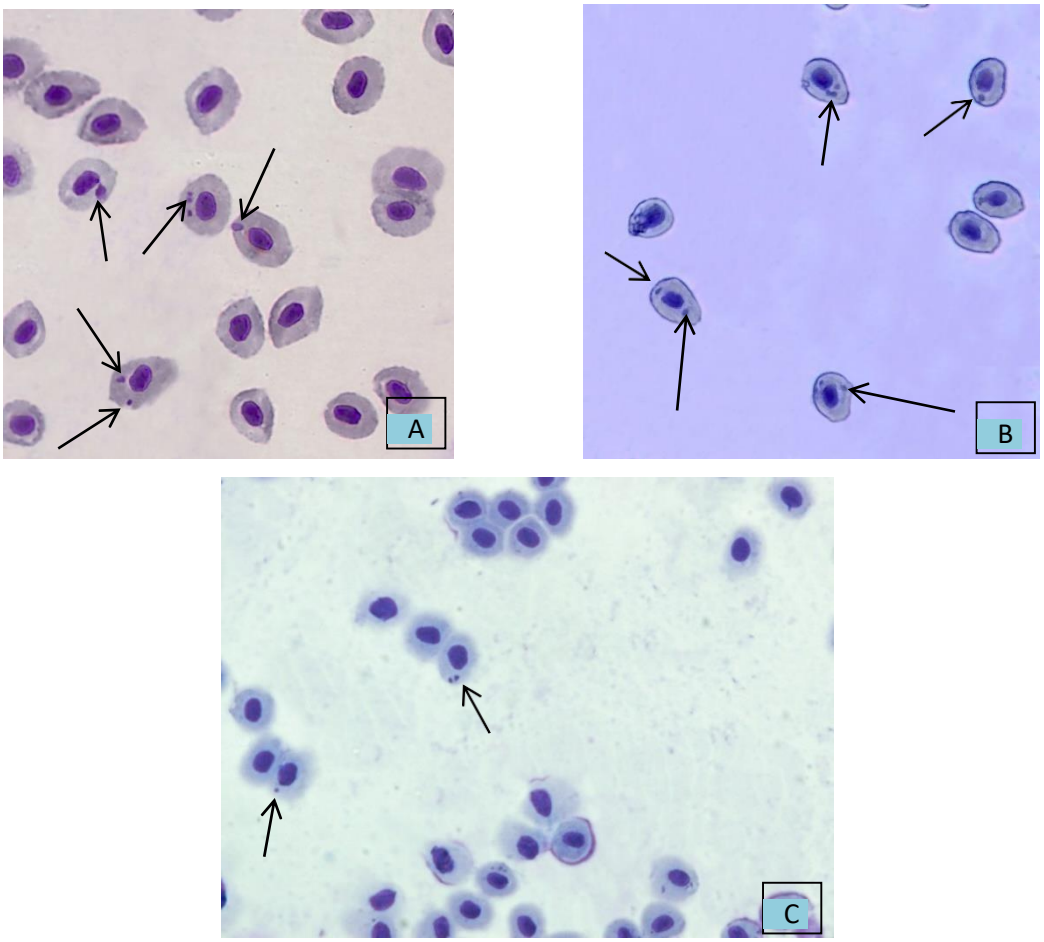


Figure 17 (A), (B), (C)-Micronucleated cells of microplastic exposed *O. mossambicus* erythrocytes

Arrows indicating micronucleated cells

PLATE VIII: Comet assay

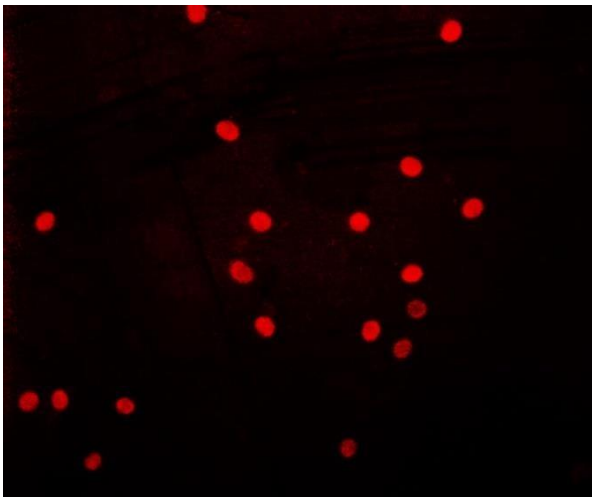


Figure 18 **Erythrocytes from *O. mossambicus* indicating normal cells**

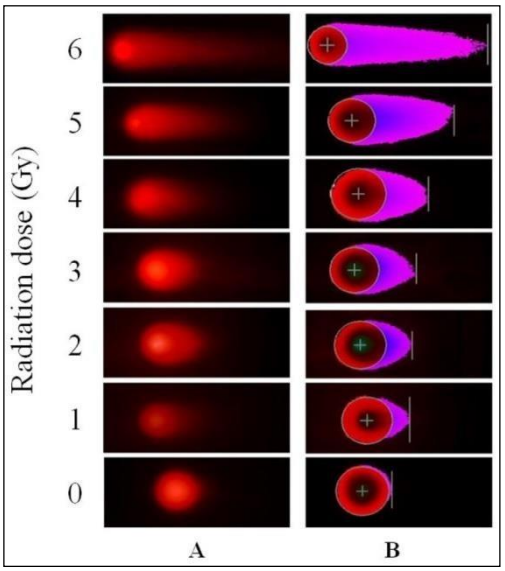


Figure 19 **Appearance of Comet Cells**

https://doi.org/10.3390/ijms141122449

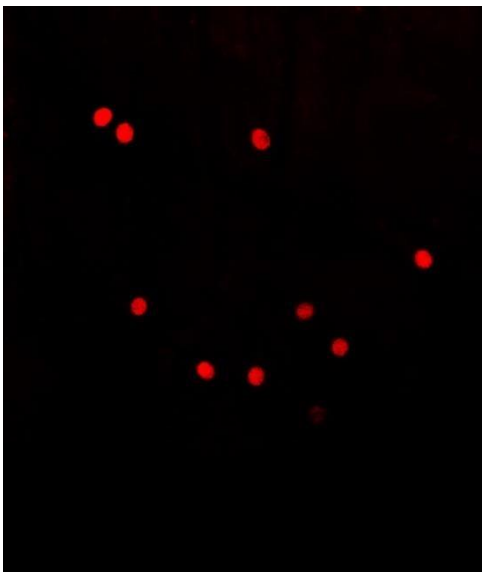


Figure 20 **Erythrocytes from *O. mossambicus* indicating normal blood cells having no DNA damage**

PLATE IX: Histology-Gill

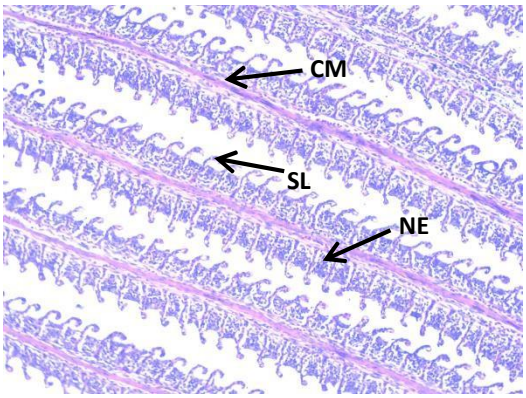


Figure 21 Histology of Gill indicating normal lamellae structures from control group at 20x magnification. CM-central matrix; SL-secondary lamellae; NE- normal epithelium

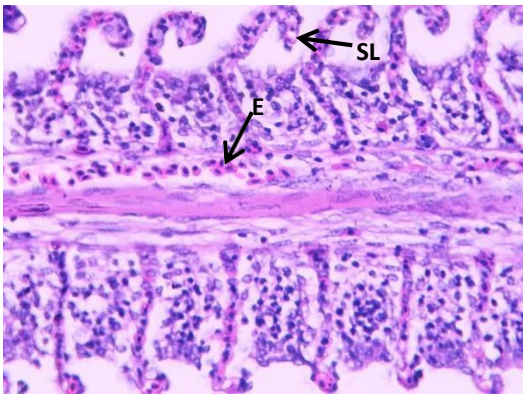


Figure 22 Histology of Gill indicating normal lamellae structure from control group at 40x magnification. SL-secondary lamellae; E-erythrocytes

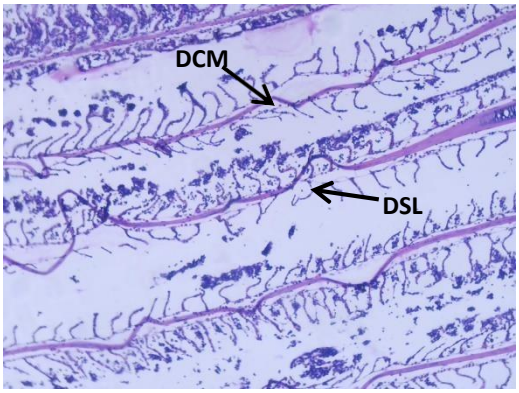


Figure 23 Histology of gill showing disrupted lamellae structure from experimental group at 20x magnification. DCM- disrupted cartilaginous matrix; DSL-disrupted secondary lamellae

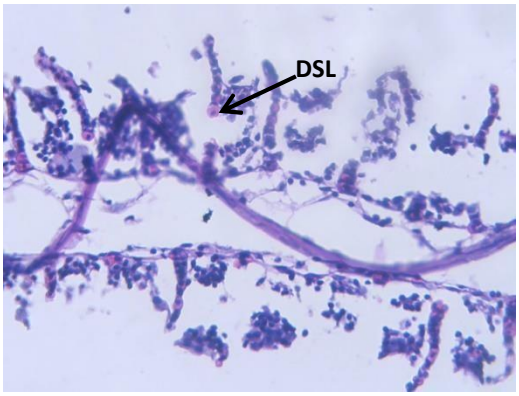


Figure 24 Histology of Gill of disrupted lamellae structure from experimental group at 40x magnification. DSL-damaged secondary lamellae

PLATE X: Histology-Gut

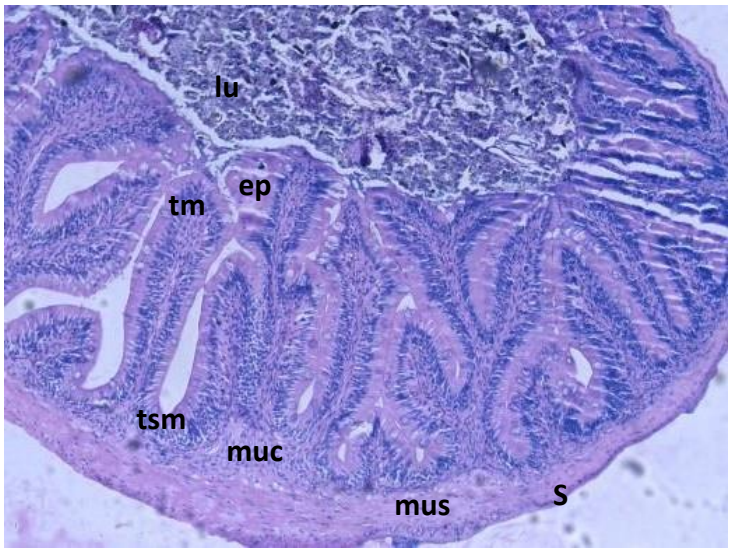


Figure 25 Histology of gut showing normal structure from control group at 40x magnification illustrating normal Villi structures. s-serosa; mus-muscularis; tm- tunica mucosa; tsm- tunica submucosa; muc-mucosa; ep-epithelium; lu-lumen

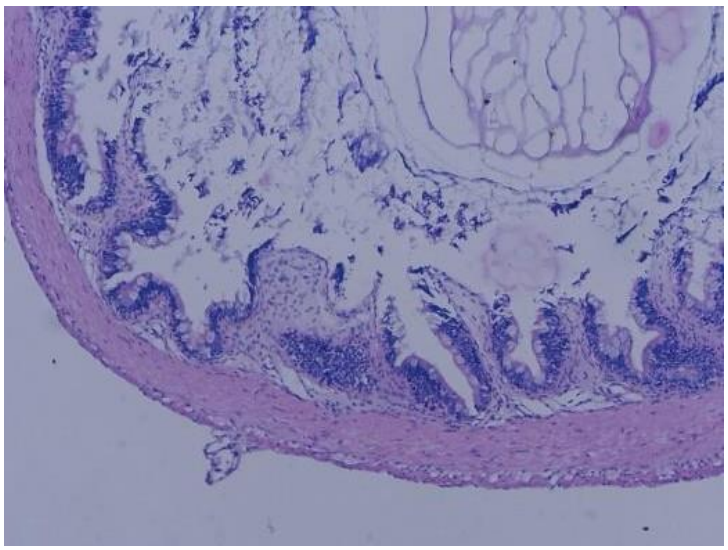


Figure 26 Histology of gut showing disruption from experimental group at 40x magnification displaying damaged villi structures (ep,tm,tsm) ep-epithelium; tm- tunica mucosa; tsm- tunica submucosa.

4.1. DISCUSSION

Exposure study was carried out by maintaining *Oreochromis mossambicus* under laboratory conditions. After the acclimatization of fish they were exposed with the Polystyrene microplastics of different concentration for an acute period of 14 days. Fish exposed to PS microplastics showed intake of PS particles in concentration dependent manner.

After being exposed to three distinct concentrations of PS microplastics for 14 days, O. mossambicus showed obvious behavioural alterations, including an initial period of hyperactivity (quick swimming, chasing, and biting other fish when they came into contact). A few fish isolated themselves in the corners of the tank and also displayed fast gasping behaviour. Following their exposure to microplastics, they displayed feeding behaviours. They expelled the particles out of their mouths after realizing they were not food. This behaviour was probably due to the energetic requirements after exposure to microplastics by the fishes for detoxification. Similar observations were noted by (Fuster *et al.,* 2021) wherein they documented behavioural changes in swimming activity of *Sparus aurata* exposed to two different microplastics for 21 days also (Raza *et al.,* 2022) found similar results of polyacrylamide exposed to O. niloticus. Zebra fish that had been exposed to microplastics exhibited more hyperactive behaviours than the fish in the control group (Chen *et al.,* 2020). Nonetheless, there are reports of behaviour linked to ingesting microplastics. According to a study, zebra fish that exhibited spitting behaviour can identify microplastic as an inedible substance (Sulaiman *et al.,* 2023).

The aim of the present study was to evaluate the accumulation and tissue distribution of PS microplastics in the *O. mossambicus*. After 14 days exposure the fishes were checked for accumulation of PS microplastics from three different concentrations. The microplastics concentrations in various tissues of *O. mossambicus* generally increased over time following the order, gill>gut>liver in concentration dependent manner. MPs can be passively consumed by foraging, or they can be actively consumed when mistaken for food (Roch *et al*., 2020). Thus, reports of MP have been made in laboratory studies as well as aquatic natural habitats (Costa *et al.,* 2020; Da Costa *et al.,* 2021). Similar results were observed by (Lu *et al.,* 2018) in zebra fish (*Danio rerio*). The likelihood of these particles being lodged in the gill filaments is increased because the gills of aquatic organisms are the first organ exposed to MPs during respiration (Gao *et al.,* 2023). A fish's intake of food is thought to be the initial point of entry for microplastics. Since the microplastics' size and color made them appear to be food, the fish in the tank actively swam toward them. The observed buildup of microplastics in the gut might be explained by this reason (Li *et al.,* 2021). Watts *et al*., (2014) report that microplastics can be ingested by common

marine nonfilter-feeding species or inspired over the gills. The fact that microplastics were absorbed through the stomach and directly into the digestive tract may account for the presence in the liver and intestines. Alternatively, it's possible that microplastics are mostly taken up primarily through the gills, where they are then carried into internal tissues by the circulatory system through the microvasculature created by the water passing through the gill cavity (Watts *et al*., 2014).

According to Hook *et al*., (2014), antioxidant biomarkers were employed to identify the effects of different environmental stresses on a variety of aquatic organisms. The current study found that the experimental groups exhibited significantly higher levels of Catalase (CAT), and Malondialdehyde (MDA) activity as compared to the control groups in a dose dependent manner in gill, gut and liver tissues. Various aquatic creatures are observed to have been affected by microplastics that caused disruption in antioxidant enzymes (Prinz and Korez 2020). Similar results were reported that suggests the activity of catalase (CAT) was increased in zebra fish (Lu *et al.,* 2016; Qiao *et al*., 2019) and *Cyprinodon variegatus* (Choi *et al*., 2018) after exposure to polystyrene microplastics. Catalase is considered as one of the enzymes of the first line defence that directly eliminate ROS. The function of Catalase is reduction of H_2O_2 to non-harmful products as water and oxygen (Prokić et al., 2019). The MDA test is commonly used as a biomarker for oxidative stress in organisms exposed to various stressors, including microplastics (Song et al., 2020). Elevated levels of MDA indicative of cellular damage caused by oxidative stress induced by microplastics, physical damage by microplastic, generation of ROS, inflammation and disruption of cellular functions (Liu et al., 2022).

Along with antioxidant biomarkers, liver functioning tests for microplastic exposed fishes were performed in gill, gut and liver tissues of *O. mossambicus*. The present study reported significant increase in concentration of Alkaline Phosphatase (ALP) and Alanine Transaminase (ALT), prominently in liver tissue. Similar results were noted by (Lee et al., 2023) where they studied toxic effects of polyethylene microplastic on *Pseudobagrus fulvidraco* indicating high levels of ALP and ALP. Additionally, (Banaei et al., 2022) found increased in ALP and ALP activities in Polyethylene microplastic treated fish Common Carp. (Hamed et al., 2019) found similar results in *O. niloticus* for microplastic toxicity. According to (Sun et al., 2024) the elevated levels of ALP and ALT might be that microplastic exposure causes overproduction of ROS in the liver leading to release of inflammatory factors and oxidative stress in fish liver.

In the present study as the concentration of microplastics increased, it was found that the content of carbohydrates and proteins were reduced and no significance was noted. (Shang et al., 2021) found similar results indicating decrease in protein and carbohydrate concentration on microplastic exposed *Mytilus coruscus.* According to (Banei et al., 2022) the breakdown of glycogen as an

energy source for countering the cytotoxic effects of MPs is responsible for the decreased accumulation of glycogen in tissues. A lower protein content could be a sign of suppressed protein synthesis as a result of decreased activity of the digestive proteases, which would impact amino acid absorption. Thus, restrictions on amino acids may have a deleterious impact on mussels exposed to microplastics' ability to synthesize proteins (Wang et al., 2020).

Histology allows for the visualization of tissue structure and characteristic changes the tissue may have undergone due to exposure of the toxicant. The current study's findings shown that, in a dose-dependent way, consumed PS microplastics considerably changed the histomorphology of gut and gill tissues There was a clear distinction in the gill tissues exposed to microplastic; there was tissue disruption overall, with disrupted lamellae structures, displaced cartilaginous matrix, and a reduction in the quantity of erythrocytes. In the gut tissue experimental group tissue samples indicated damaged structures due to PS microplastic exposure resulting in damaged villi and distortion of epithelial lining. (Lu et al., 2018; Yang et al., 2020 and Hamed et al., 2021) made similar observations. (Sayed et al., 2024) state that the group subjected to PS-NPs had severe histological changes in their gills as well as structural cell damage where PS-NP accumulation had taken place. According to Hu et al., (2020), the gill alterations could have resulted from either mechanical harm, reactions to leached chemicals, or a mix of both factors. As a defensive measure to keep oneself away from toxins, the separation of epithelium from the basal membrane is a sign of osmoregulation abnormalities (Movahedinia et al., 2012). Similarly, an overall decrease in surface area for gas exchange results from the fusing of lamellae (Farrell et al., 2010). Furthermore, Karami et al., (2016) hypothesized that the damage was likely caused by the sharper texture of particular MPs. Because of its large surface area and continuous, permanent intake of water from the surrounding environment during breathing, gills are particularly vulnerable to all kinds of toxicants (Hawkins et al., 2015). It functions as a very trustworthy bio-indicator of water pollution as a result. According to (Mobin et al., 2000) disruption of the epithelium layer may permit cytotoxic agents from the intestinal lumen to reach the bloodstream, which could lead to functional changes in a number of internal organs. The concluded that the presence of MPs in the small intestine results in histological lesions, impairs energy metabolism and nutritional availability, and significantly worsens fish welfare (Qiao et al., 2019).

Chromosome breakage and DNA strand damage were examined using genotoxicity tests, such as the micronucleus test and comet assay test, on fish exposed to PS microplastics and fish in the control group. A significant difference was seen between the control group and the three experimental groups in the micronucleus test, with an F value of $F=343.1$ and $p<0.0001$. Similar outcomes in Mytilus galloprovincialis (mussels) exposed to polyethylene and polystyrene

microplastics were reported by Avio et al., (2015). As established markers of genotoxic damage, the Micronucleus, Nuclear Buds, and Blebbed Nuclei Cells rose dramatically in *S. trutta* exposed to MPs, according to Jakubowska et al., 2020. Fish erythrocytes showed an increase in total genotoxicity (∑Gentox) in the following order: PS>PET>PE (Jakubowska et al., 2020). The following study, which linked oxidative stress in tilapia with exposure to microplastic, was carried out by Hamad et al., 2020 and revealed that the genotoxicity of polyethylene microplastic on erythrocytes was indirectly caused by the generation of free radicals that interfere with DNA integrity. Additionally, (Hamad et al., 2021) reported nucleus abnormalities such as micronucleus by notched, lobed and blebbed nuclei for microplastics exposed to early juvenile *O. niloticus*. DNA strand breaks were anticipated to be the initial type of damage caused on by the increased production of ROS in reaction to MPs. When compared to other MPs, a higher PS-induced prooxidant response might cause permanent DNA integrity loss (nuclear abnormalities), which elevates the frequency of micronuclei (Avio et al., 2015).

The comet assay was used to evaluate DNA damage in comet-shaped cells. In both single and double standards, the comet assay aids in the identification of DNA damage. In the current investigation, there was no significant difference found between the experimental and control groups. It can be inferred from the lack of considerable variance between control and experimental samples that the exposed fish under study did not exhibit breakage in their DNA strands. Freire et al., (2023) noted similar findings when they examined the toxicity of polyethylene microplastic in zebra fish (Danio rerio). According to the results, there were no DNA breaks in the comet assay test when the tail length of the nucleoids was examined for all exposure levels. Based on an acute exposure analysis, PS microplastic larger than nanoparticles was used in the current study. According to (Brandts et al., 2022) since, nanoparticles were examined and showed more significant results for DNA break damage for chronic exposure than microplastics, it is possible that the plastic's particle size contributed to the negative outcomes.

4.2. CONCLUSION

The present study was conducted on the toxic effects of Polystyrene microplastics on laboratory maintained *O. mossambicus* (Tilapia). After the acclimatizing the fishes, the fishes were subjected to different concentrations for the evaluation of LC50 study.

Since no LC50 was found three different concentration were decided as 50 mg/L, 250 mg/L and 500mg/L for 14 days exposure period. Accumulation of PS microplastics was seen in three different tissues in following order: Gill>Gut>Liver. Toxicological tests such as Catalase assay, TBARS assay for oxidative stress, ALP and ALT test for liver function tests and protein, carbohydrates as nutrient profiling tests were performed on three tissues i.e., gill, gut and liver of *O. mossambicus*.. Along with this genotoxicity tests such as Micronucleus test and Comet assay with Histopathological studies were carried out on subsequent model organism. The results of toxicological tests helped to monitor the health of the *O. mossambicus* exposed to PS microplastics by showing variable toxicity responses. This indicates that *O. mossambicus* are sensitive to higher concentrations of microplastics in the environment. Toxicological tests carried out for exposed fishes showed higher concentrations of Catalase, MDA, ALP, ALT and lower concentrations of Protein and Carbohydrates. For genotoxicity tests Micronucleus test showed significant increase in micronucleated cells compared to the control group but in contrast, Comet assay showed no significant observations. Histology of gill and gut tissues indicated significant alterations caused due to Polystyrene microplastics.

As *O. mossambicus* plays important role as economically important fish, accumulation and biomagnification of microplastic pollutants in the trophic level will have detrimental effects on the health of higher organisms including humans.

4.3. FUTURE SCOPE

The current research focuses on the harmful impacts of Polystyrene microplastics on Oreochromis mossambicus. It revealed that these microplastics are toxic to Oreochromis mossambicus as evidences by significant differences in biomolecular, biochemical and genotoxicity tests between control and experimental groups. Moving forward potential future avenues are:

1. Investigating toxic effects of chronic exposure of Polystyrene microplastics.
2. Evaluation of effects of Polystyrene microplastics on freshwater organisms.
3. Comparative exposure study of primary and secondary Polystyrene microplastics.
4. Evaluation of effects of Polystyrene microplastics after depuration.

CHAPTER 5

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