The alarming impact of microplastics on the reproductive system of Fish

Submitted by:

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An Internship Project Report Submitted by:

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Certificate of Summer Internship

This is to certify that Mr. Sayak Ghosh, a student of Haldia Institute of Technology has satisfactorily completed his two months summer internship in my laboratory at Presidency University on "Alarming effect of microplastics on the reproductive system of fish" from July to August 2023. I was truly impressed by his enthusiasm to explore the impact of microplastics on the molecular mechanism of oocyte maturation and steroid production in zebrafish. In addition to this, he has learnt several techniques like DNA/RNA isolation, ELISA, PCR, Western blotting, SDS-PAGE etc during this internship.

Sayak has keen interest in reading scientific journal and develop his own research ideas. He is obedient, honest and sincere person and works great as a team. It was a pleasure to have him in laboratory. I wish her all the best in his future research pursuits.

Prof. Kousik Pramanick
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Abstract

Environmental contaminants that are biologically active significantly affect ecosystems, animals, and human health. For the preservation of biodiversity and the health of ecosystems, plastic pollution has gained international attention. The aquatic ecology of our planet is full of microplastics. Regular, extensive use of nonbiodegradable polymers required several years for them to break down. Over the course of those years, it discharged tiny particles of 100 nm or less. Due to their size resemblance to Particular Organic Matter, these microplastics (MPs) are frequently ingested by the smaller species present in the waterbody, such as zooplankton and shellfish. The top-level organism in that food chain was affected by this process. Then MPs infiltrated the body and disrupted their ability to do their regular tasks. In this work, we exposed the model fish *Danio rerio* to MP treated water taken from neighbouring plastic-polluted water bodies to examine the impact of microplastics on its reproductive system. Another water tank is prepared using the commercially available polystyrene MPs mixing with the water. To assess the MPs' potential impact, we also employ fluorescent PS-MPs. This study shed light on the reproductive harm that microplastics in fish may do and has ramifications for environmental toxicology and freshwater ecology.

Keywords: microplastics, reproduction, fertility, fish reproduction, environmental toxicology

1. Introduction

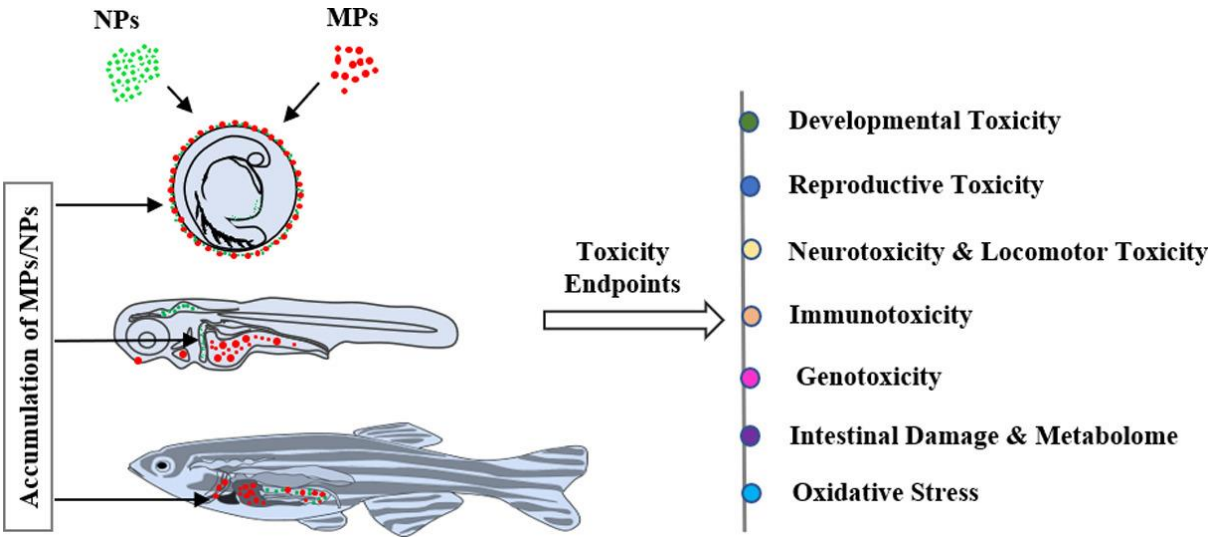
Plastic Pollution is a growing concern for the world's aquatic system. It has been documented in both the fresh and marine water environment. Plastics have many uses due to its low cost, high durability and lightweight. There are two main source of plastics pollution - household and the industry. Use plastics are dump in landfill or in the barren land along with the normal waste. Only about 5% of plastic products are recycled but most of the plastics end up in landfills and oceans each year (North & Halden, 2013). These large plastic items undergo fragmentation through UV exposure, oxidation, hydrolysis, wave action and other degradation processes (Hidalgo-Ruz *et al.*, 2013). These degradation process leads to the formation of microplastic particles that are less than 5 mm in the longest dimension. These MPs are constantly mixing with the freshwater and the marine water through the outlets of industry, drains of city etc. This issue is more prominent in the fresh water ecosystems. Importance of freshwater ecosystem is well-defined. 40% of fish in the world came from the fresh water. These habitats of fishes are facing the large amount discharge of MPs.

Microplastics (MPs) are defined by as "synthetic solid particles or polymeric matrices, with regular or irregular shape and with size ranging from 1 μm to 5 mm, of either primary or secondary manufacturing origin, which are insoluble in water." Microplastics are frequently present in freshwaters and drinking water, and number concentrations spanned ten orders of magnitude across individual samples and water types. The primary sources of MPs may be plastic production and/or recycling processes as well as daily activities such as using of personal care products (face wash gels, toothpaste, shower gels) and washing machines (Browne et al., 2007). The secondary MPs are released from the degradation of plastics found in marine litter, sanitary landfills, industrial or agricultural sources via mechanical decomposition, photolysis, thermal decomposition, thermo-oxidation, and biodegradation.

Due to having the similar structure of food fish engulf the MPs and process runs throughout the food chain. Zooplankton organisms are the primary consumers in the food chain and are important food for aquaculture and fisheries. Studies revealed the possible hazards of plastic particles in the food chain, such as plankton acting as potential carriers of toxic substances derived from the ingestion of plastic particles (Teuten et al., 2009). Fish are important players of the aquatic environments. Bony fish like zebrafish occupy higher trophic levels in the food web and play the essential role of transporting energy and matter and sustaining ecosystem stability. MPs have been documented in the digestive system of wild-caught fish and the aquatic invertebrates (Foekema et al., 2013; Phillips and Bonner, 2015; Cole et al., 2011). We use PS in this study because this polymer is one of the main products that produce in high value. 90% of total plastic's demand is fulfilled by PS and also use in aquatic culture, fishing activities.

Once consumed, microplastics can remain in the digestive tracts of that organism for period of days to weeks before excretion. Microplastic reproductive toxicity was reported to be closely related to oxidative damage (Xie *et al*., 2020). According to Wang et al. (2019) that exposure to 20 mg/L of 10 mm polystyrene microplastics for 60 days disrupted reproductive endocrine system and caused histological changes in gills and testes. Some study reveal that the polymer has significant effects on animal development such as defect in intestine, decreased body size, lowering the survival rate, neurotoxicity and metabolism defect. Through this study we are going to reveal the effects of MPs

on reproduction and reproductive system of fish, *Danio rerio.* Further in this article we declared why we use this fish as our organism to test the impact of MPs.



Graphical Representation of how MPs and NPs Effect the fish (Bhagat et al. 2020)

As previously told that in this study we use wild type of zebrafish both male and female. We purchased those fish from local supplier. There are some main characterise to use zebrafish as a test organism.

Genetic Similarity: Zebrafish share many genetic similarities with other vertebrates, including humans. Approximately 70% of human genes associated with diseases have a zebrafish counterpart. This similarity makes zebrafish a valuable model for understanding genetic mechanisms and studying human diseases.

Transparent Embryos: One of the key advantages of zebrafish is that their embryos are transparent, allowing researchers to observe the developmental processes in real-time. This transparency simplifies the study of organ development and cell behaviour, providing valuable insights into various biological processes.

Rapid Development: Zebrafish have a short generation time and develop rapidly. They are capable of reaching reproductive maturity within a few months, and their embryos develop from fertilization to hatching within just a few days. This quick development facilitates time-efficient studies, making it possible to observe multiple generations in a relatively short period.

Large Number of Offspring: Zebrafish produce a large number of offspring in each mating, providing researchers with ample specimens for experimentation. This high fecundity ensures that researchers have a substantial sample size, improving the reliability of their findings.

Easy Maintenance: Zebrafish are relatively easy to maintain in the laboratory. They require less space and resources compared to other model organisms like mice or rats. This cost-effectiveness makes them a preferred choice for large-scale experiments and screening studies.

Well-Characterized Genome: The zebrafish genome has been extensively sequenced and characterized, making it a valuable resource for genetic studies. This knowledge facilitates genetic manipulation and targeted experiments.

Regenerative Abilities: Zebrafish have a remarkable ability to regenerate various tissues and organs, such as their fins and heart. Studying this regenerative capacity can provide insights into potential regenerative therapies for humans.

Pharmacological Studies: Zebrafish can efficiently absorb small molecules through their skin and gills, making them suitable for drug screening studies. Researchers can use zebrafish to test potential drug candidates and assess their effects on different biological processes.

Fig 1: *A pair of zebra fish in the water tank. Male fish are a bit smaller than the female fish.*

2. Objective

- The objective of our work is to find the effect of microplastics on the reproductive system of fish or in the reproductive system of zebra fish *(danio rerio).*
- Histological Examination of ovary to find the effect of MPs
- To find the hormonal activity: Luteinizing Hormone and Follicle Stimulating Hormone in presence of MPs.
- Molecular mechanism of toxicity on gene expression level.

3. Materials and Methods

In this study we use different type of materials and instrument on the purpose to fulfil the project objective. First, we come to the materials

3.1) Animals

Wild Type of Zebrafish (around 6 months old) were purchased from a local supplier. Those 3 – 4 cm long fishes are acclimated in laboratory aquaria (50 L capacity: Size – 30"x18"x15") for around 2 weeks at proper temperature around 26 ± 1° C with a photoperiod of 14:10 h light/dark. They were fed with commercial fish food (Shalimar fish food, Bird and Fish food manufacturer, Mumbai) twice a day. After acclimation the fish was exposed to the water mixed with polystyrene micro beads. They are exposed in the toxic water for the next 30 days.

3.2) Chemicals

Polystyrene microspheres with a diameter of 1 μm is taken. It is commercially available in Thermo Fisher Scientific (FluoSpheres Polystyrene, Concentration 1 x 10^{10} beads⁄mL). 4 types of concentration were used in this study – 0 μg/L, 10 μg/L, 25 μg/L and 50 μg/L.

We use s tricaine methane sulfonate (MS-222) as an anesthetic for the experimental fish. Total RNA isolation (TRI) reagent was purchased from Ambion (Foster City, CA, USA). cDNA synthesis kit purchased from Biorad. Eva green SYBER mix (Biorad), eosin, methyl benzoate, haematoxylin was used in different step of this experiment. All other chemicals used were of analytical grade.

Now come to the instruments part, we need every minute instruments to big instruments like micro pipette, measuring cylinder, Petri dish, slides, scalpel, scissors, PCR blocks, RT-PCR machine, microscopes, water bath, refrigerator, centrifuge, cryostat microtome, weighing balance etc.

Materials	**Instruments and miscellaneous**
• Zebrafish • Polystyrene as MPs • Tricaine MS-222 • RNA extraction kit • cDNA kit • SYBER mix • Ethanol • Eosin • Haematoxylin	• Autoclave • Laminar Air Flow • Glass bead steriliser. • Precision balance • Micropipette • Tissue Paper • Petri Dish. • Lighter • Forceps and Scalpel • Scissors

• Para Formaldehyde	• Beaker • Conical flask • Measuring cylinder • Spatula • Microtome • qRT PCR

3.3) *In Vivo* Experiment

For *in vivo* experiment, initially we divided the fishes into four (4) different groups. 1st group contain 5 fishes and others groups are contain 10 fishes both male and female. Group 1 is exposed in normal water. Group 2,3,4 is exposed in 10 µg/L, 25 µg/L and 50 µg/L MPs treated water respectively and separately. We consider the normal water fish as the control for this experiment. They are exposed in this treated water for 30 days. After 30 days fishes from each group are collected and sacrificed for the sample collection. Fish were anesthetized with MS 222 and dissected to collect the blood, liver, testis and ovary. Blood is collected Eppendorf tube, levelled and stored in refrigerator. Liver, Testis and ovary are kept in paraformaldehyde for further experiments like histology and immunohistochemistry.

Fig 2: *Fishes are kept in treated water*

3.4) Gonadosomatic and Hepatosomatic index

Gonadosomatic index = gonad weight (testis for male and ovary for female) / Total Body weight and Hepatosomatic index = weight of liver / Total body weight. After dissecting the fish, we separate the liver, testis and the ovaries. Fish gonad somatic index rises as they mature, reaching its maximum at their peak time of maturity, and then it drastically drops after spawning. Liver is a vital organ of any living organism which control and regulate the metabolism. In case of fish, it regulates the vitellogenesis. Vitellogenesis is a process of yolk protein formation in oocyte. It is mostly observed in non-mammalian vertebrates during sexual maturation. Vitellogenin production in liver is the first step of vitellogenesis. If MPs damage the Liver, which we can calculate using the HPI, will negatively impact in yolk formation.

3.5) RNA isolation and cDNA synthesis

Total RNA from ovarian follicles was isolated using TRI reagent (Promega) following the manufacturer's instruction and the method described earlier. Quantification of RNA was performed in a Nanodrop spectrophotometer and cDNA synthesis was carried out with 2.0 µg of DNase-treated

total RNA using Revert Aid M-MuLVreverse transcriptase (MBI; Fermentas, USA) and a mix of oligo(dT) and random primers (Promega), according to the manufacturer's protocols. RNA and cDNA were stored at -70°C and -20°C, respectively.

3.6) RT quantitative Polymerase Chain Reaction to study gene expression

In fish, sexual development and maturation are controlled by many sex hormones in the HPG axis, such as gonadotropins (GTH), gonadotropin-releasing hormone (GnRH), follicle-stimulating hormone (FSH), and luteinizing hormone (LH) (Ma et al., 2012; Cao et al., 2019). Gonadotropic hormones are then transported to the gonads to cause steroidogenesis producing sex steroid hormones which regulate the reproductive process and disturb hypothalamic and pituitary regulatory activities through feedback mechanisms. Gonadotropic hormones are then transported to the gonads to cause steroidogenesis producing sex steroid hormones which regulate the reproductive process and disturb hypothalamic and pituitary regulatory activities through feedback mechanisms (Nagahama and Yamashita, 2008). ERs play momentous roles in sexual differentiation and maturation, such as testicular development, oogenesis, and vitellogenesis (Ishibashi and Kawashima, 2001; Kim et al., 2014). By using the Primer 3 software (Whitehead Institute for Biomedical Research) both forward and reverse primers were designed from a partial mRNA sequence of *Danio rerio.* The total reaction volume was 20 µl contained 10 µl of SYBR Green, 5µl of cDNA and 500nM of forward and reverse primers. Reactions were run in a Bio-Rad PCR system maintaining following protocol: 50 ºC for 2 min, 96 ºC for 10 min, 45 cycles of 95 ºC for 30 s and 62 ºC for 1 min. All samples were run in triplicate.

3.7) Histological study of Liver, Testes and Ovary

Isolated Tissues, which were kept in paraformaldehyde, are transferred in liquid wax and left in the incubator for some time. Then the tissues are ready to prepare block. We use Leica Microtome to cut the tissues block. They are place on the slide using hot water bath. For the histological study H&E stain is the common staining procedure. H&E contains the two dyes haematoxylin and eosin. Eosin is an acidic stain which stain the cytoplasm and haematoxylin is a basic stain which stain the nucleus. Xylene is used in this staining procedure to remove the wax from the slide and D.P.X. is used to mount the cover slip. Then prepared slide is ready to observe under the microscope.

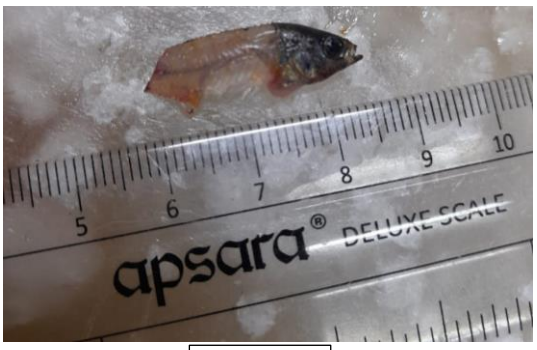


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Fig 3: *After dissection of a fish measuring the length of it;* ***Fig 4:*** *Microtome to cut the prepared block into slide form;* ***Fig 5:*** *Slides are prepared for staining and observation under microscope*

3.8) Steroid Extraction from Blood

Fresh blood from is collected from different sample and stored in Eppendorf tube. Then the blood is mixed with diethyl ether and vortex it well. Eppendorf tube is now left for dry in open air to evaporate the ether. This extract will be mixed with ELISA buffer further.

3.9) Enzyme Linked Immunosorbent Assay

In this experiment we perform ELISA to detect specific protein in blood serum, like: LH, FSH. The effort looking at how microplastics affect fish reproductive systems uses ELISA as a key instrument. Our knowledge of the detrimental effects of microplastic pollution on aquatic ecosystems is improved by its capacity to quantitatively measure biomarkers, evaluate endocrine disruption, provide comparative analysis, and offer insights into long-term effects, which emphasises the urgency of mitigating its effects.

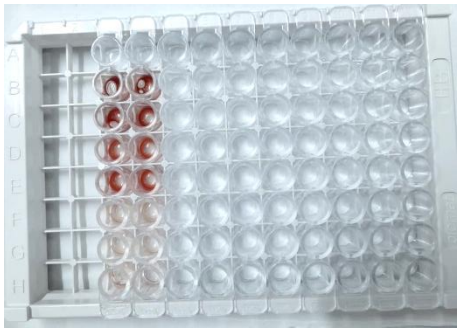

Fig 6: *Performing ELISA with fish blood serum*

4. Results and Discussion

We did every possible test from that we could collect data and state the effect of MPs on Fish reproduction system. But here we focus on the Histological quantification and identification. We carefully measured the GSI and HIS.

4.1) No effect on survival rate of *Danio rerio*

As we can observe in this study, for 30 days of toxin exposer even in the highest value survival rate is 100 percent. Not a single was decreased in this period. The intensity of MPs was increasing in individual body of fish with the days.

4.2) Effects of MPs on the quantification of histology and gonadal morphology

In the control females, there were a large proportion of primary and secondary follicles in the ovary. After 15 days of exposure, the proportion of primary follicles in the ovary of fish exposed to MPs was significantly decreased compared with the controls. The proportion of primordial follicles was increased by 12.81%, while the proportion of primary follicles, secondary follicles, and mature follicles were reduced by 17.64%, 12.91%, and 28.33% in ovaries of fish respectively to the higher concentration of MPs. Relative to the controls, the proportion of mature follicles was significantly decreased by 20% in ovaries. Moreover, the proportion of secondary follicles and mature follicles in ovary was obviously reduced.

In the control testes, different stages of germ cells were found in the lobular cavity of seminiferous lobules. After 15 days of exposure, the proportion of spermatogonia was significantly decreased by 36.73% in testes of fish compared with the controls. The proportion of spermatozoa was

dramatically reduced by 46.63% in testes of fish compared to the controls. After 30 days of exposure, the proportion of spermatocytes and spermatozoa in testes of fish exposed to 50 µg/L of MPs was significantly decreased by 88.03%.

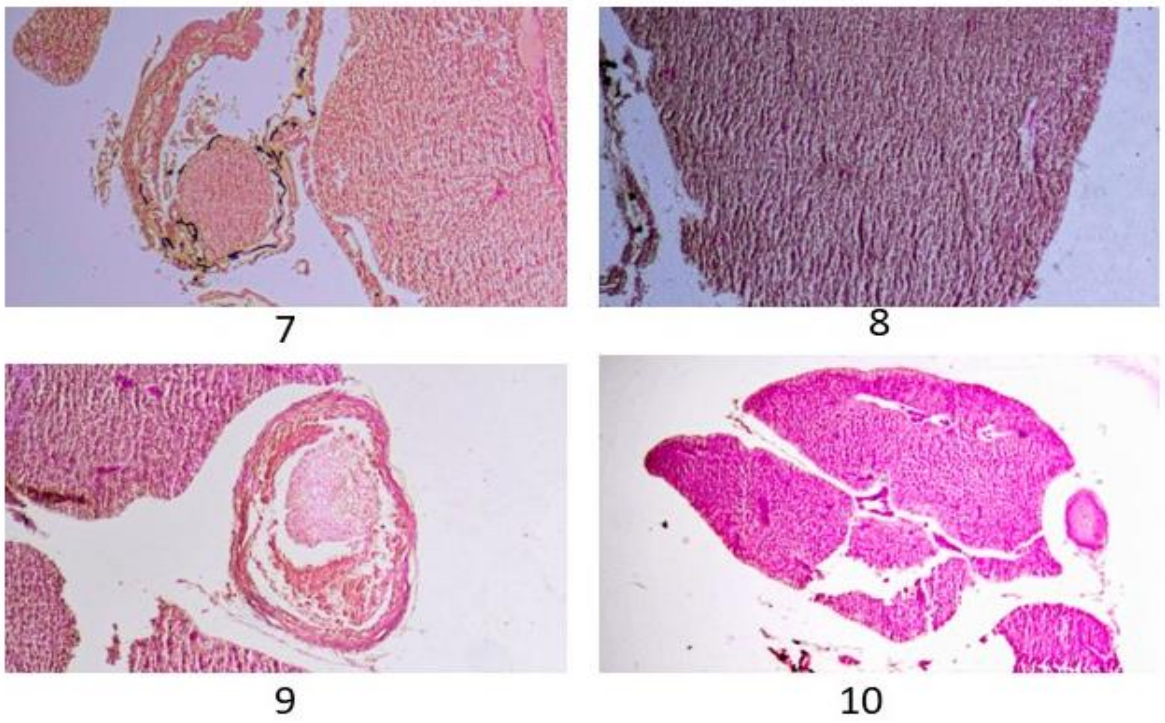

Fig 7, 8, 9, 10 : *Histological Microscopic Image of Fish Liver*

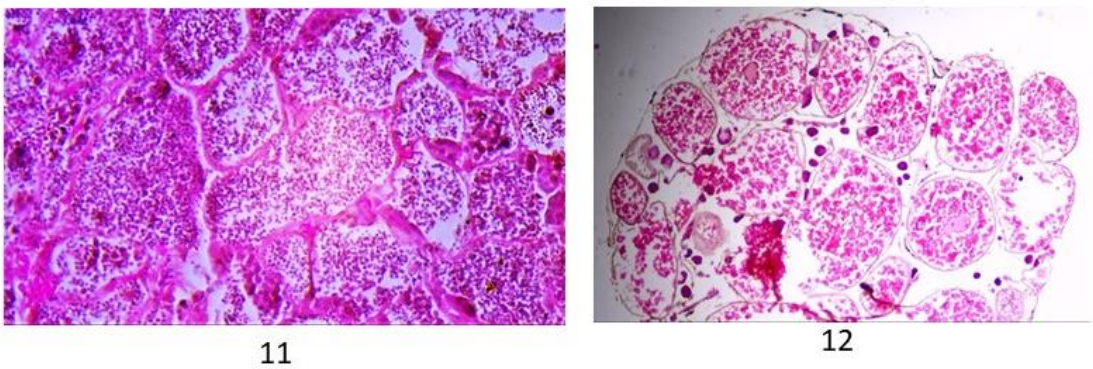

Fig 11: *Histological Microscopic Image of Testis* ***Fig 12:*** *Histological Microscopic Image of Ovary*

4.3) MPs Ingestion Induced Delayed Sexual Maturity

The effect of exposure to MPs led to an increase in the number of days of birth to clutching of eggs in fishes compared to the control group and this change is higher in high concentration. Effects on the population and the days of sexual maturity in D. magna under exposure of MPs. After MP treatment of zebra fish females, 20 newborn neonates were transferred to and incubated in MP-free

freshwater for 30 days. The *D. Rerio* offspring showed fast sexual maturity with 3 days under freshwater (no MPs) and released their eggs at 5th day. However, the other offspring derived from MP-treated Zebra Fish presented delayed sexual maturity (over 4 days) and offspring production (6th day). All the tests included 3 independent replicates.

4.3) Effect of MPs on Reproductive hormones

During ovarian and follicle development, both FSHR and LHR have low expression in the follicles of primary growth stage (PG or stage I). However, when the PG follicles are recruited to initiate vitelologenesis (PV, previtellogenic or stage II), there is a significant increase in FSHR but not LHR expression. The expression of LHR significantly increases in the mid-vitellogenic stage (MV, or mid-stage III) and peaks at the full-grown (FG) stage. These results suggest that the two gonadotropin receptors have distinct functions in folliculogenesis , with FSHR being involved in follicle recruitment and vitellogenic growth and LHR being important in the later stages of follicle development including final oocyte maturation.

4.4) The Changes in `GSI and HIS

As microplastics are intake by fishes in every concentration, the effect of it is shown differently according to the concentration. We can tell that no such effect in 10 microgram/L Fishes on their GSI but the Liver of the fishes are decreased in size. To investigate the molecular impact of polystyrene microplastics exposure, ROS levels of both female and male brain, liver and gonad tissues, represented by respective average optical density (AOD) values obtained, were examined for both the control (0 microg/L) and polystyrene microplastic treatment groups (10, 20 and 50 microg/L) after 30 days. In the testis, the area of immature spermatocytes (spermatogonia and spermatocytes) and mature spermatocytes (spermatids and spermatozoa) were calculated to reflect the ratio of spermatocytes at different developmental stages (Qiang & Cheng, 2021).

No significant difference in the ratio of spermatocytes at different developmental stages was observed in the zebrafish testis upon exposure to polystyrene microplastics when compared to the control. However, the basement membrane thickness of zebrafish testis was observed to have significantly decreased by 0.173 mm when exposed to 50 microg/L of polystyrene microplastic (2.700 ± 0.081 mm) compared to the control group (2.873 ± 0.073 mm). Male fish showed further histological alterations in response to polystyrene microplastic exposure in this study. The secondary organs of fish reproductive systems, which include the basement membrane, are essential factors of reproductive fitness. The basement membrane provides alimentation and supports the structural and functional integrity of the blood-testis barrier and of the testis; impairment of the basement membrane will severely compromise the function of the testis (Ogedengbe et al., 2018). Male fish can be more sensitive than females to environmental pressures.

5. Conclusion

The project highlights the alarming impact of microplastics on fish's reproductive systems, highlighting the need for comprehensive actions to mitigate the issue. Microplastic exposure causes endocrine disruption, impaired reproductive performance, altered gene expression, and compromised fertility. The implications extend beyond the aquatic ecosystem and potentially affect human health and the environment. A multi-faceted approach involving policymakers, industries, researchers, and communities is required. Regulation and reduction of plastic production, improved

waste management systems, and eco-friendly alternatives are critical steps. Further research is needed to understand the mechanisms underlying microplastics' impact on fish reproductive systems. Long-term monitoring and assessment programs will provide valuable insights into intervention effectiveness and ecosystem recovery. Recognizing the interconnectedness of all living organisms and ecosystems, responsible consumption, and environmental stewardship are essential. By working towards solutions, we can restore ocean health and safeguard fish's reproductive health, ensuring a sustainable future for nature and humanity. This Project is still under going examination and experiment. So, result may vary with the final and appropriate outcome.

6. References

1. Bhagat, J., Zang, L., Nishimura, N., & Shimada, Y. (2020). Zebrafish: An emerging model to study microplastic and nanoplastic toxicity. Science of the Total Environment, 728, 138707. https://doi.org/10.1016/j.scitotenv.2020.138707
2. Browne, M. A., Galloway, T., & Thompson, R. (2007). Microplastic--an emerging contaminant of potential concern? *Integrated environmental assessment and management*, *3*(4), 559–561. https://doi.org/10.1002/ieam.5630030412
3. Cao, J., Wang, G., Wang, T., Chen, J., Guo, W., Wu, P., He, X., & Xie, L. (2019). Copper caused reproductive endocrine disruption in zebrafish (Danio rerio). *Aquatic Toxicology*, *211*, 124–136. https://doi.org/10.1016/j.aquatox.2019.04.003
4. Cole, M., Lindeque, P. K., Halsband, C., & Galloway, T. S. (2011). Microplastics as contaminants in the marine environment: A review. *Marine Pollution Bulletin*, *62*(12), 2588–2597. https://doi.org/10.1016/j.marpolbul.2011.09.025
5. Foekema, E., De Gruijter, C., Mergia, M. T., Van Franeker, J., Murk, A. J., & Koelmans, A. A. (2013). Plastic in North Sea fish. *Environmental Science & Technology*, *47*(15), 8818–8824. https://doi.org/10.1021/es400931b
6. Guchhait, R., Chatterjee, A., Gupta, S., Debnath, M., Mukherjee, D., & Pramanick, K. (2018). Molecular mechanism of mercury-induced reproductive impairments in banded gourami, Trichogaster fasciata. *General and Comparative Endocrinology*, *255*, 40–48. https://doi.org/10.1016/j.ygcen.2017.10.004
7. Hidalgo-Ruz, V., Gutow, L., Thompson, R. C., & Thiel, M. (2012). Microplastics in the Marine Environment: A review of the methods used for identification and quantification. *Environmental Science & Technology*, *46*(6), 3060–3075. https://doi.org/10.1021/es2031505
8. Ji, K., Hong, S., Kho, Y., & Choi, K. (2013). Effects of bisphenol s exposure on endocrine functions and reproduction of zebrafish. Environmental Science & Technology, 47(15), 8793–8800. https://doi.org/10.1021/es400329t
9. Kim, N. N., Shin, H. S., Choi, Y. J., & Choi, C. Y. (2014). Kisspeptin regulates the hypothalamus–pituitary–gonad axis gene expression during sexual maturation in the cinnamon clownfish, Amphiprion melanopus. *Comparative Biochemistry and Physiology B*, *168*, 19–32. https://doi.org/10.1016/j.cbpb.2013.11.002
10. Ma, Y., Han, J., Guo, Y., Lam, P. K., Wu, R. S., Giesy, J. P., Zhang, X., & Zhou, B. (2012). Disruption of endocrine function in in vitro H295R cell-based and in in vivo assay in zebrafish by 2,4-dichlorophenol. *Aquatic Toxicology*, *106–107*, 173–181. https://doi.org/10.1016/j.aquatox.2011.11.006
11. North, E. & Halden, R. (2013). Plastics and environmental health: the road ahead. *Reviews on Environmental Health*, *28*(1), 1-8. https://doi.org/10.1515/reveh-2012-0030
12. Ogedengbe, O. O., Jegede, A. I., Onanuga, I. O., Offor, U., Peter, A. I., Akang, E. E. U., Naidu, E. C., & Azu, O. O. (2017). Adjuvant potential of virgin coconut oil extract on antiretroviral therapy-induced testicular toxicity: An ultrastructural study. Andrologia, 50(3), e12930. https://doi.org/10.1111/and.12930

13. Phillips, M., & Bonner, T. H. (2015). Occurrence and amount of microplastic ingested by fishes in watersheds of the Gulf of Mexico. *Marine Pollution Bulletin*, *100*(1), 264–269. https://doi.org/10.1016/j.marpolbul.2015.08.041
14. Teuten, E. L., Saquing, J. M., Knappe, D. R., Barlaz, M. A., Jonsson, S., Björn, A., Rowland, S. J., Thompson, R. C., Galloway, T. S., Yamashita, R., Ochi, D., Watanuki, Y., Moore, C. J., Viet, P. H., Tana, T. S., Prudente, M. S., Boonyatumanond, R., Zakaria, M. P., Akkhavong, K., . . . Takada, H. (2009). Transport and release of chemicals from plastics to the environment and to wildlife. *Philosophical Transactions of the Royal Society B*, *364*(1526), 2027–2045. https://doi.org/10.1098/rstb.2008.0284
15. Wang, J., Li, Y., Lu, L., Zheng, M., Zhang, X., Tian, H., Wang, W., & Ru, S. (2019). Polystyrene microplastics cause tissue damages, sex-specific reproductive disruption and transgenerational effects in marine medaka (Oryzias melastigma). *Environmental Pollution*, *254*, 113024. https://doi.org/10.1016/j.envpol.2019.113024
16. Xie, X., Deng, T., Duan, J., Xie, J., Yuan, J., & Chen, M. (2020). Exposure to polystyrene microplastics causes reproductive toxicity through oxidative stress and activation of the p38 MAPK signalling pathway. *Ecotoxicology and Environmental Safety*, *190*, 110133. https://doi.org/10.1016/j.ecoenv.2019.110133