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# The Alarming Impact of Microplastics on the Reproductive System of Fish

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***Abstract***

*Environmental contaminants that are biologically active significantly affect ecosystems, animals, and human health. Plastic pollution has drawn international attention for its detrimental effect on the environment and biodiversity health. 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 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 neighboring 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 mixed 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

## INTRODUCTION

Plastic Pollution is a growing concern in aquatic systems worldwide. It has been recorded in both freshwater and seawater. Plastics have many uses owing to their low cost, durability, lightweight, and ease of availability. There are two main sources of plastic pollution: household and industrial. Plastics are dumped in landfills or barren land, along with normal waste. Approximately 5% of plastic products are recycled, but most plastics end up in landfills and water bodies, such as oceans and lakes, each year [1]. These large plastics undergo fragmentation through UV exposure, oxidation, hydrolysis, wave action, and other degradation processes [2]. These degradation processes lead to the formation of microplastic particles less than 5 mm in the longest dimension. These MPs constantly mix with freshwater and marine water through the outlets of industry, drains of cities, etc. This issue is particularly prominent in freshwater ecosystems. The importance of freshwater ecosystems has been well-defined. Forty percent of the fish in the world come from fresh water. These fish habitats are facing a large amount of discharge of MPs [3].

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Microplastics, defined as synthetic particles or polymer matrices, range in size from 1 μm to 5 mm and are insoluble in water. They can be either primary, originating from manufacturing processes, or secondary, formed from the breakdown of larger plastics. These particles are commonly found in freshwater systems and drinking water, with concentrations varying widely, depending on the sample and water type. The major sources of microplastics include plastic production and recycling activities, as well as everyday activities

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such as using personal care products (such as face washes, toothpaste, and shower gels) and laundry processes [4]. The breakdown of plastics found in sanitary landfills, industrial or agricultural sources, marine litter, photolysis, thermal decomposition, thermal oxidation, and biodegradation releases secondary MPs [5].

Due to the similar structure of food, fish engulf MPs and the process runs throughout the food chain. Zooplankton are the primary consumers in the food chain and are important food sources for aquatic animals and fisheries. Research has demonstrated the potential risks associated with plastic particles in the food chain, including the possibility that plankton can become hazardous substances when they swallow plastic particles [6]. Fish are a significant participant in aquatic ecosystems. Bony fish-like zebrafish occupy higher trophic levels in the food web and play an essential role in transporting energy and matter and sustaining ecosystem stability. MPs have been documented in the digestive systems of wild-caught fish and aquatic invertebrates [7–9]. We used PS in this study because this polymer is one of the main products that produce a high value. Approximately 90% of the total plastic demand is fulfilled by PS, which is used in aquatic culture and fishing activities.

Once consumed, microplastics can remain in the digestive system of an organism for a period of days to weeks before excretion. Microplastic reproductive toxicity is closely associated with oxidative damage [10]. According to a study [11], exposure to 20 mg/L of 10 mm polystyrene microplastics for 60 days disrupted the reproductive endocrine system and caused histological changes in the gills and testes. Some studies have revealed that polymers have significant effects on animal development, such as defects in the intestine, decreased body size, reduced survival rate, neurotoxicity, and metabolic defects. In this study, we aimed to reveal the effects of MPs on the reproductive system of *D. rerio*. Further, in this article, we declare why we used this fish as our organism to test the impact of MPs.

As previously mentioned, in this study, we used wild-type zebrafish, both male and female. The fish were purchased from a local supplier. There are some main characteristics of using zebrafish as a test organism.

- *Genetic similarity:* Zebrafish share many genetic similarities with other vertebrates, including humans. Approximately 70% of the human genes associated with diseases have zebrafish counterparts. This similarity makes the 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 developmental processes in real time. This transparency simplifies the study of organ development and cell behavior, providing valuable insights into various biological processes.
- *Rapid development:* Zebrafish have a short generation time, and their growth is rapid compared to that of other fish in this category. They can reach reproductive maturity within a few months, and their embryos develop from fertilization to hatching within a few days. This rapid growth promotes time-efficient investigations, allowing the observation of numerous generations in a very short timeframe.
- *Large number of offspring:* Zebrafish exhibit prolific reproductive capacity, yielding a considerable number of progenies in each breeding cycle. This elevated fecundity provides researchers with a plentiful supply of specimens, thereby enhancing the statistical power and robustness of their experimental outcomes.
- *Easy maintenance:* Zebrafish have relatively low maintenance in a laboratory setting, requiring minimal space and resources compared to other model organisms such as mice or rats. This economic nature makes them an ideal choice for extensive experiments and high-throughput screening studies.
- *Well-characterized genome:* The zebrafish genome has been extensively sequenced and characterized, making it a valuable resource for genetic studies. This knowledge will facilitate genetic manipulation and targeted experiments.

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

## MATERIAL AND METHOD

In this study, we used different types of materials and instruments to fulfill the project objectives. First, we discuss the materials used.

- *Animals:* Wild-type zebrafish (approximately six months old) were purchased from a local supplier. Those 3–4 cm long fishes are acclimated in laboratory aquaria (50 L capacity; Size 30″×18″×15″) for around 2 weeks at a proper temperature of around 26 ± 1°C with a photoperiod of 14:10 h light/dark. The fish food was administered twice a day. After acclimation, the fish were exposed to water-containing polystyrene microbeads. They were then exposed to toxic water for 30 days.
- *Chemicals:* Polystyrene microspheres with a diameter of 1 μm were obtained. It is commercially available from Thermo Fisher Scientific (FluoSpheres Polystyrene, Concentration 1 × 1010 beads⁄mL). Four different concentrations were used in this study: 0, 10, 25, and 50 μg/L. Tricaine methane sulfonate (MS-222) was used as an anesthetic for the experimental fish. Total RNA isolation (TRI) reagent was purchased from Promega. A cDNA synthesis kit was purchased from Bio-Rad. Eva green SYBER mix (Bio-Rad), eosin, methyl benzoate, and hematoxylin were used in different steps 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 such as micropipettes, measuring cylinders, Petri dishes, slides, scalpels, scissors, PCR blocks, RT-PCR machines, microscopes, water bath, refrigerator, centrifuge, cryostat microtome, and weighing balance.
- *In vivo experiment:* For vivo experiment, the fish were initially divided into four different groups. 1st group contained five fish, and the other groups contained 10 male and female fish. The rats in group 1 were exposed to normal water. Groups 2, 3, and 4 were exposed to 10, 25, and 50 μg/L MPs, respectively. We used normal water fish as the control for this experiment. They were then exposed to treated water for 30 days. After 30 days, the fish from each group were collected and sacrificed for sample collection. Fish were anesthetized with MS 222 and dissected to collect blood, liver, testis, and ovaries. Blood was collected in Eppendorf tubes, leveled, and stored in a refrigerator. The liver, testis, and ovary were kept in paraformaldehyde for further histological and immunohistochemical experiments.
- *RNA isolation and cDNA synthesis*: Total RNA from ovarian follicles was isolated using the TRI reagent (Promega). We followed the manufacturer's instructions and a previously described method. RNA was quantified using a Nanodrop spectrophotometer, and 2.0 μg of DNase-treated total RNA was used for cDNA synthesis using RevertAid M-MuLV reverse transcriptase (MBI Fermentas, USA), along with a combination of random primers and oligo(dT) from Promega, in accordance with the manufacturer's instructions. RNA and cDNA were stored at -70°C and -20°C, respectively.
- *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) [12, 13]. Gonads receive gonadotropic hormones that induce steroidogenesis and produce sex steroid hormones. These hormones control reproduction and interfere with pituitary and hypothalamic regulatory functions via feedback loops. Gonads receive gonadotropic hormones, which induce steroidogenesis and produce sex steroid hormones that control reproduction and interfere with pituitary and hypothalamus regulatory functions via feedback processes [14]. ERs play significant roles in sexual differentiation and maturation, including oogenesis, vitellogenesis, and testicular development [5]. Using Primer 3 software (Whitehead Institute for Biomedical Research), forward and reverse primers were designed from

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a partial mRNA sequence of *Danio rerio*. The total reaction volume was 20 µL, containing 10 µL SYBR Green, and 5 µl µL cDNA. Reactions were run in a Bio-Rad PCR system using the following protocol: 50°C for 2 min, 96°C for 10 min, 45 cycles of 95°C for 30 s, and 62°C for 1 min. All samples were run in triplicates.

- *Histological study of liver, testes, and ovary*: Isolated tissues, which were kept in paraformaldehyde, were transferred to liquid wax, and left in the incubator for some time. The tissues were then ready to prepare a block. A Leica Microtome was used to cut the tissue blocks. They were placed on a slide in a hot water bath. For the histological study H&E stain is the common staining procedure. H&E contains two dyes, hematoxylin, and eosin. Eosin is an acidic stain that stains the cytoplasm and hematoxylin is a basic stain that stains the nucleus. Xylene was used in this staining procedure to remove wax from the slide, and D.P.X. was used to mount the cover slip. The prepared slide was then ready to be observed under a microscope.
- *Steroid extraction from blood*: Fresh blood was collected from different samples and stored in Eppendorf tubes. Blood was then mixed with diethyl ether and vortexed. The Eppendorf tube was left to dry in open air to evaporate the ether. The extract was further mixed with the ELISA buffer.
- *Enzyme-linked immunosorbent Assay:* In this experiment, we performed ELISA to detect specific proteins in the blood serum, such as LH and FSH. ELISA is a key instrument to examine how microplastics affect fish reproductive systems. 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, emphasizing the urgency of mitigating its effects.

## RESULTS AND DISCUSSION

We performed every possible test from which we could collect data and determine the effect of MPs on the fish reproduction system. However, here we focus on histological quantification and identification. We carefully measured the GSI and HIS scores.

1. *No effect on the survival rate of Danio rerio*: As observed in this study, for 30 days of toxin exposure, even at the highest survival rate of 100 percent. There was not a single decrease in this period. The intensity of MPs increased in the bodies of individual fish over time.
2. *Effects of MPs on the quantification of histology and gonadal morphology*: In control females, there was a large proportion of primary and secondary follicles in the ovary. After 15 days of exposure, the proportion of primary follicles in the ovaries of fish exposed to MPs was significantly lower than that in the controls. The proportion of primordial follicles was increased by 12.81%, while the proportion of primary follicles, secondary follicles, and mature follicles was reduced by 15.64%, 12.91%, and 26.33% in the ovaries of fish, respectively, in response to a higher concentration of MPs. In the ovaries, the percentage of developed follicles was considerably reduced by 22% compared to that in the controls. Moreover, the proportion of secondary and mature follicles in the ovaries was reduced. In control testes, germ cells at different stages were found in the lobular cavity of the seminiferous lobules. After 15 days of exposure, the proportion of spermatogonia was significantly decreased by 33.73% in the testes of fish compared to that in the controls. The proportion of spermatozoa in fish testes drastically decreased by 45.85% compared with the standards. After 30 days of exposure, the proportion of spermatocytes and spermatozoa in the testes of fish exposed to 50 µg/L MPs significantly decreased by 88.03%. The ingestion of MPs delays the development of sexuality. MP exposure led to an increase in the number of days from birth to clutches of eggs in fish relative to the control group, and this shift was more pronounced at high concentrations. The impact of MP exposure on the population and the number of days that D. magna reached sexual maturity. After MP treatment of zebrafish females, 20 newborns were transferred and incubated in MP-free water for 30 days. The *D. rerio* offspring showed fast sexual maturity within 3 days in freshwater and released their eggs on the 5th day. The other offspring from the MP-treated zebra fish, on the other hand, showed delayed sexual maturation (over 4 days) and sixth-day offspring production. For every test, there were typically three independent replicates.

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3. *Effect of MPs on reproductive hormones*: During ovarian and follicle development, both FSHR and LHR show low expression in the follicles of the primary growth stage (PG or stage I). However, there is a notable increase in FSHR expression, but not LHR expression, when PV, previtellogenic, or stage II vitellogenesis is initiated by the recruitment of PG follicles. The expression of LHR significantly increased in the mid-vitellogenic stage (MV, or mid-stage III) and peaked 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. *The changes in GSI and HIS*: As microplastics are consumed by fish at every concentration, their effects vary according to the concentration. No such effect was observed in 10 µg/L fish on their GSI but the Liver of the fish was decreased in size. To examine the molecular effects of exposure to polystyrene microplastics, the average optical density (AOD) values of the brain, liver, and gonad tissues in male and female subjects were used to reflect the ROS levels in each tissue and were examined for both the control (0 µg/L) and polystyrene microplastic treatment groups (10, 20, and 50 µg/L) after 30 days. To represent the ratio of spermatocytes at various developmental stages, the area of immature spermatocytes (spermatogonia and spermatocytes) and mature spermatocytes (spermatids and spermatozoa) in the testes was calculated [15]. No significant difference in the ratio of spermatocytes at different developmental stages was observed in the zebrafish testis upon exposure to polystyrene microplastics compared to the control. However, the basement membrane thickness of zebrafish testes was observed to have significantly decreased by 0.173 mm when exposed to 50 µg/L of polystyrene microplastic (2.700 ± 0.081 mm) compared to the control group (2.873 ± 0.073 mm). In this study, male fish exposed to polystyrene microplastic displayed further histological changes. The basement membrane, a secondary organ of the fish reproductive system, is a crucial component of reproductive fitness. According to Ogedengbe et al. (2018) [16], damage to the basement membrane significantly affects testis function. The basement membrane supplies nourishment and maintains the structural and functional integrity of the testis and blood-testis barrier. Male fish may be more susceptible to environmental stress than are female fish.

## CONCLUSION

The initiative draws attention to the worrying effects that microplastics have on fish reproductive systems and emphasizes the necessity of taking comprehensive steps to address the problem. Microplastic exposure causes endocrine disruption, reduced fertility, altered gene expression, and poor reproductive function. These consequences could have an impact on the environment and human health in addition to aquatic ecosystems. It is necessary to adopt a multifaceted approach by engaging communities, businesses, scholars, and legislators. Important measures include enhanced 16 waste management systems, regulation and decrease of plastic production, and the use of eco-friendly substitutes. The processes underlying the effects of microplastics on fish reproductive systems require further investigation. Programs for long-term monitoring and assessment will offer insightful information about the success of interventions and ecosystem restoration. Understanding the interdependence of ecosystems and all living things, practicing environmental care, and judicious consumption are crucial. By working towards solutions, we can restore ocean health and safeguard fish reproductive health, ensuring a sustainable future for nature and humanity.

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