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RESEARCH PAPER

Effects of Microplastics on the Activity of Digestive-, Antioxidative- and Immune-related Enzymes in Soiny Mullet (*Liza haematocheila* Temminck & Schlegel, 1845) Larvae

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Abstract

Microplastics (MPs), novel pollutants in the water environment, can cause damage to fish, especially to fish larvae. However, the effects of MPs on fish in estuarine areas with high levels of MPs pollution are unknown. In this study, a 14-day exposure test was conducted to analyze the effects of MPs on soiny mullet larvae by measuring the activities of digestive enzymes, antioxidant enzymes, and immune enzymes in soiny mullet larvae after exposure to MPs of different particle sizes (0.5 µm, 0.1mg/L; 6 µm, 0.1mg/L).Results showed that MPs exposure significantly decreased the activities of pepsin (PPS), alpha-amylase (α-AMS), catalase (CAT), and superoxide dismutase (SOD), and significantly increased the activities of acid phosphatase (ACP) and alkaline phosphatase (ALP), among which the PPS, CAT, SOD, ACP, and ALP were more sensitive to exposure to small particle size MPs (0.5 µm).Overall, exposure to MPs reduced digestion, destroyed the antioxidant system, and triggered an immune response in soiny mullet larvae, especially the small particles of MPs (0.5 µm). The results provide basis for understanding the detriment of microplastic in fish.

Introduction

Plastic products are widely used in all aspects of daily life due to their cheapness and durability (Sui et al., 2020). From the 1950s to 2018, global plastic production has risen from 1.5 million tons to 359 million tons (Dobaradaran et al., 2018). However, the recycling rate of all plastics is only 1% -5%, and the final dis<charge into the ocean accounts for 10% of the total plastic production (Güven et al., 2017; Mattsson et al., 2017; Tata et al., 2020). Plastics are decomposed into microplastics (MPs) with a diameter of less than 5 mm under long-term biological, physical and chemical effects (Thompson et al., 2004; Andrady, 2011). At present, MPs pollution in the ocean and rivers has attracted worldwide attention.

Estuaries are generally considered to be the main passageway for MPs to enter the ocean from freshwater environments and it is also a hot spot for investigating MPs pollution in recent years (Zhang et al., 2019). Research in China shows that the average concentration of MPs in Haihe Estuary and Yongdingxinhe Estuary surface water samples were respectively 1485.7±819.9 items/m^3 and 788.0±464.2 items/m^3 (Wu et al., 2019).

The abundance of MPs in Jiaojiang, Oujiang, and Minjiang estuaries in Zhejiang ranged from 100 items/m^3 to 4100 items/m^3 (Zhao et al., 2015). Yangtze Estuary MPs abundance was 4137.3±2461.5 items/m^3 (Zhao et al., 2014). The MPs pollution is much more serious at China's Pearl River Estuary, reaching 8902 items/m^3 (Yan et al., 2019). Now, the MPs pollution in the estuarine areas is becoming an environmental problem all over the world. However, current studies mainly focus on the pollution degree of MPs in the estuarine areas, limited research studies the effects of MPs on the large of animals in the estuarine areas (e.g. physical or immune status).

The choice of polystyrene for this study stems from its extensive use across various products, making it a significant contributor to the substantial amount of plastic entering our oceans. Its widespread prevalence underscores importance in the context of environmental pollution (Hanachi et al., 2021). MPs can be ingested by a variety of marine animals, including crustaceans (Murray & Cowie, 2011; Devriese et al., 2015), mollusks (Van Cauwenberghe and Janssen, 2014), marine mammals (Fossi et al.,2012; Bravo Rebolledo et al., 2013), zooplankton species (Desforges et al., 2015), marine turtles (Tourinho et al., 2010), sea birds (Bravo Rebolledo et al., 2013), and fish (Pedà et al., 2016; Rummel et al., 2016; Steer et al., 2017). Polystyrene may be excreted in the feces and enter the bloodstream after absorption through the intestinal epithelium and distributed to other tissues in the body. The particles may penetrate cell membranes and enter the fish. Once MPs are absorbed into the digestive system, they can penetrate the anaerobic tissues of the intestine and accumulate in the fatty tissues of the animal (Banaei et al., 2022; Kim et al., 2021). Small size MPs can enter the circulatory system and accumulate in various tissues under certain conditions (Browne et al., 2008). Studies indicate that environmental stress and ingestion of toxic substances can cause oxidative stress and affect fish digestion and immune functions, thereby inducing changes in the activity of these enzymes (Couillard et al., 2009; Blewett & Leonard, 2017; Zhao et al., 2022). The resulting biological consequences can affect the growth and development, reproduction ability, and survival rate of marine animals (Mazurais et al., 2015; Nobre et al., 2015; Sussarellu et al., 2016; Beiras et al., 2018). During the fish larvae stage, organ development and related physiological functions are influenced by many external factors, which determine the ability to cope with the challenges of survival in the following physiological stages (Shahriari Moghadam et al., 2014). The healthy growth of fish larvae is critical to the sustainable development of fish stocks and the stability of ecosystems (Steer et al., 2017). Therefore, it is particularly important to explore the negative effects of MPs on larvae fish.

Soiny mullet (*Liza haematocheila* Temminck & Schlegel, 1845) is an important farmed fish in China (Qi et al., 2022). As migratory fish, soiny mullet larvae live in the estuaries with dramatic environmental changes, such as MPs pollution, N_2O pollution, metal pollution, and ocean acidification (Soletchnik et al., 2007; Murray et al., 2015; Wijesiriet al., 2019). Compared with adult fish, fish larvae are more sensitive to the changes of the external environment and easier to be observed (Yang et al., 2020). Therefore, by taking fish larvae as the test object, we aimed to observe the impact of external stimuli on the body in detail. As filter-feeding fish, soiny mullet larvae can inadvertently eat toxic MPs. Furthermore, this study aims to explore the effect of exposure to polystyrene (PS) MPs with different particle sizes (6 µm and 0.5 µm) for 14 d on the soiny mullet larvae under laboratory conditions. We stimulated the extreme environments by using the abundance of 0.1 mg/L MPs in both treatment groups. The activity of digestive enzymes, antioxidant enzymes, and immune enzymes, including pepsin (PPS), alpha-amylase (α-AMS), catalase (CAT), superoxide dismutase (SOD), acid phosphatase (ACP), and alkaline phosphatase (ALP) were analyzed, providing the theoretical basis for further research on the effects of MPs on fish, especially larvae fish.

Materials and Methods

Soiny Mullet Preparation

A total of 1,000 healthy soiny mullet larvae (average body length 2.67±0.16 cm, average body weight 0.14±0.01g) were provided by a local fish farm in Sheyangtown, Yancheng city, Jiangsu province, China. In the laboratory, the fish were reared for at least one week in artificial seawater simulating pristine water conditions (temperature=25°C, salinity=15‰, pH=8.1, dissolved oxygen>7.0 mg/L) to acclimate. The fish were fed with brine shrimp (*Artemia salina* Linnaeus, 1758) twice daily at the rate of 2% of the fish's body weight. Excrement and uneaten food were removed by suction on a daily basis.

MPs Exposure

The monodisperse PS microspheres were purchased from BaseLine ChromTech Research Centre (Tianjin, China). Based on the size of the fish being studied and the size of the microplastics that are more common in the environment in previous studies, 0.5 µm (2.5 w/v, 10 ml, 0.5±0.012 µm, Figure 1) and 6 µm (2.5 w/v, 10 ml, 6±0.031 µm, Figure 2) MPs were chosen in this study and added to the seawater(Huang et al., 2020; Le et al., 2018). To simulate extreme MPs pollution in the water environment, the concentration of MPs in the experimental group was set to be 0.1 mg/L (Yan et al., 2020). During the experimental period, the aerators and air stones were used to maintain the suspension of MPs.

Three treated groups were set up in this study, which was named as blank control group (CTRL), small particle size MPs (0.5 µm) treated group (SMPs), and large particle size MPs (6 μm) treated group (LMPs), respectively. Each treated group contained 3 replicated glass tanks (20 fish per tank). During the 14 d test period, the artificial seawater was completely replaced daily to to guarantee the constant concentration of microplastics.

Sample Collection

All the following experimental methods were under the National Institutes of Health Guide for the Care and Use of Laboratory Animals of China.

At the 3rd, 7th, and 14th day post exposure (dpe), 3 fish were randomly selected from each tank for sampling. Before sampling, the soiny mullet larvae were euthanized through anesthetic overdose with tricaine methane-sulfonate (MS-222) (Sigma, USA). Sterile scissors and tweezers to cut off the head and tail of the larvae, and the remains were transferred the remainder into a cryovial. In order to handle the samples in a uniform manner to ensure the reliability of the experiments and the accuracy of the results, the cryovials were quickly frozen in liquid nitrogen and transferred to -80°C for storage until the biochemical analyses began. The FTIR analysis was used to confirm that microplastic has been removed by the exposed larvae.

Enzymes Activities Assay

Each sample was homogenized in normal saline (0.9%) in a 1:10 weight: volume ratio. After high-speed centrifugation, the supernatant was absorbed for the measurement of different enzyme activities. Mixed them well, incubated at 37°C for 20 minutes, and read at 450 nm. The amount of enzyme corresponding to 50% SOD inhibition in this reaction system is one SOD activity unit (U) and the unit of enzyme is U/mgprot. The protein concentration of each sample was determined by Coomassie brilliant blue method. Afterward, the activities of PPS, α-AMS, CAT, SOD, ACP, and ALP were measured on a microplate reader, respectively. All enzymes activities kits in this study were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). We strictly followed the operation steps and calculation formula of the kit's instructions to determine the activities of each enzyme.

Statistical Analyses

The data in the study were tested for homogeneity of variances and normal distribution first. The data significant differences were analyzed using one-way or multiple-way analysis of variance (ANOVA) followed by Tukey's post hoc test to evaluate the differences in the

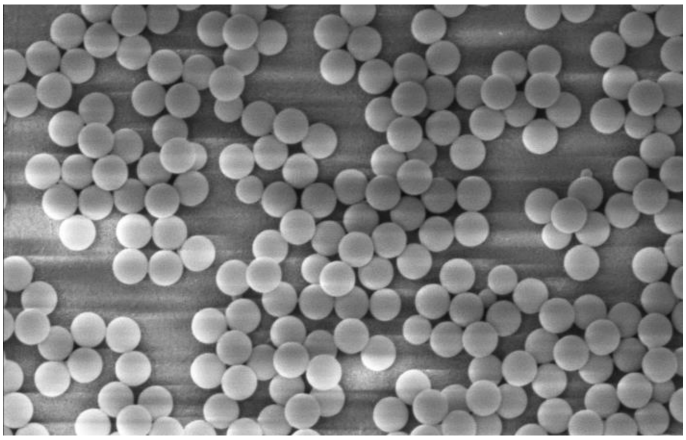

Figure 1. Microscopic image of 0.5 μm MPs.

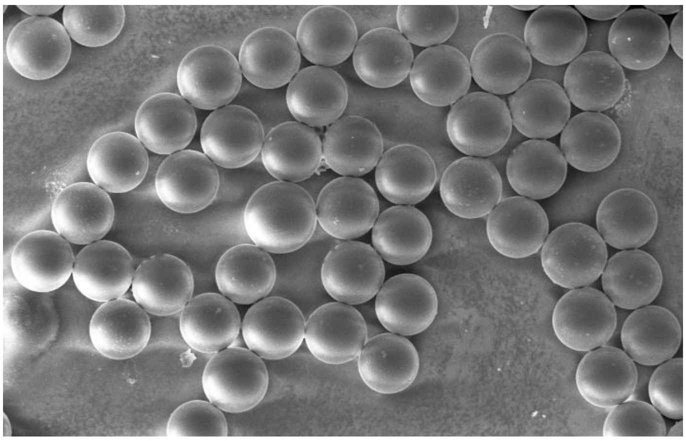

Figure 2. Microscopic image of 6 μm MPs.

influence of MPs size and MPs exposure time on test indexes. The data difference was considered significant and had statistical significance when P<0.05. The statistical analysis of the data was carried out by the SPSS 24.0 statistical software (Chicago, USA). All results were expressed as mean±standard deviation (SD).

Results

Effects of MPs on the Activities of Digestive Enzymes of Soiny Mullet Larvae

The effect of MPs exposure on the PPS activities of soiny mullet was shown in Figure 3. The MPs exposure reduced the activity of PPS, especially in SMPs. However, the enzyme activities of the two treated groups remained stable throughout the experimental period. The PPS activity of LMPs and SMPs were significantly lower than that of CTRL (P<0.05). In addition, the PPS activity of SMPs was significantly lower than that of LMPs (P<0.05) (Figure 3).

The effect of MPs exposure on the α-AMS activities of soiny mullet was shown in Figure 4. At 3 dpe, the MPs exposure had no significant effect on the α-AMS activity (P>0.05). At 7 dpe, MPs exposure did not show no significant effect on the α-AMS activity of SMPs (P>0.05), either, but significantly inhibited the the α-AMS activity in LMPs (P<0.05). At 14 dpe, the MPs exposure significantly reduced the α-AMS activity in SMPs (P<0.05), and also significantly inhibited the α-AMS activity in LMPs, which was lower than these in the CTRL and SMPs (P<0.05) (Figure 4).

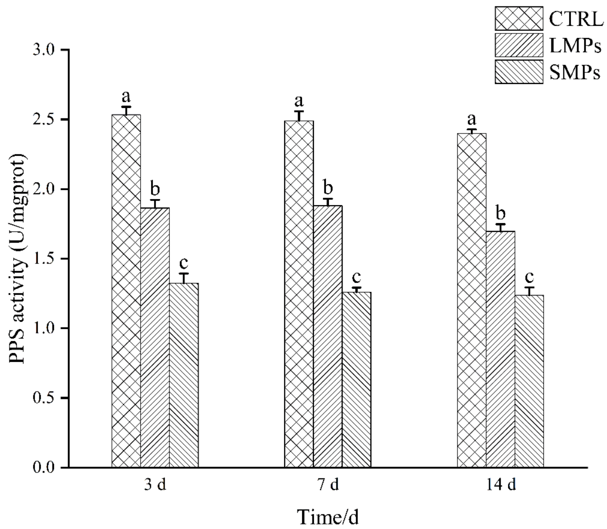

Figure 3. Effects of exposure to MPs with different particle sizes on PPS activity of soiny mullet (*Liza haematocheila*) larvae (n=9). Data were presented as mean ± SD. Different letters indicate significant differences (P<0.05). Conversely, there is no significant difference between the same letters.

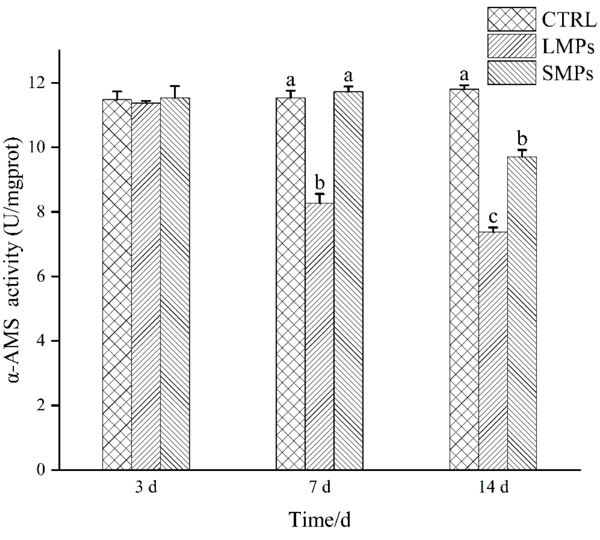

Figure 4. Effects of exposure to MPs with different particle sizes on α-AMS activity of soiny mullet (*Liza haematocheila*) larvae (n=9). Data were presented as mean ± SD. Different letters indicate significant differences (P<0.05). Conversely, there is no significant difference between the same letters.

Effects of MPs on the Activities of Antioxidative Enzymes of Soiny Mullet Larvae

The effect of MPs exposure on the SOD activities of soiny mullet was shown in Figure 5. At 3 dpe, the SOD activity in SMPs was significantly lower than that in CTRL (P<0.05). However, no significant difference was observed for the SOD activity between the LMPs and CTRL (P>0.05). At 7 and 14 dpe, the MPs exposure significantly reduced the SOD activity in LMPs and SMPs (P<0.05)(Figure 5). The SOD activity reached lowest values in the LMPs, compared with that of CTRL and SMPs (P<0.05).

The MPs exposure slightly increased the CAT activity in LMPs at 3 dpe, but shared no significant change with other groups (P>0.05). However, the CAT activity in SMPs was inhibited and significantly lower than that in the LMPs and CTRL (P<0.05). At 7 dpe and 14 dpe, the CAT activities of SMPs and LMPs were significantly lower than that of CTRL (P<0.05) (Figure 6).

Effects MPs on the Activities of Immune Enzymes of Soiny Mullet Larvae

The effect of MPs exposure on the ACP activities of soiny mullet was shown in Figure 7. MPs exposure significantly induced the ACP activities throughout the test period, compared with that of CTRL (P<0.05). As shown in Figure 7, the ACP activity in SMPs was significantly higher than that of LMPs (P<0.05), indicating that the ACP activity was mostly affected by the small particle size MPs.

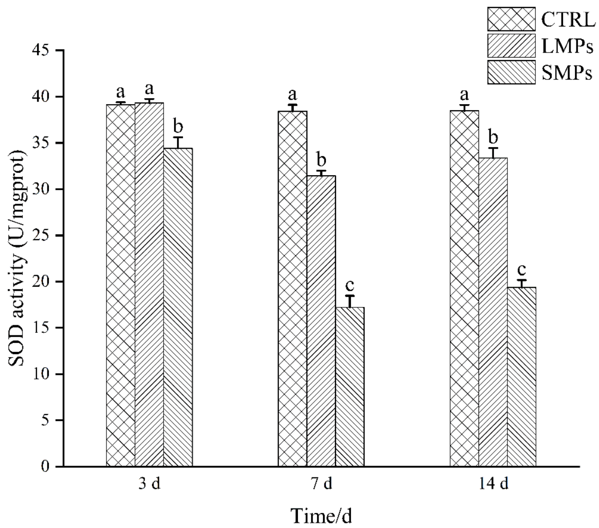

Figure 5. Effects of exposure to MPs with different particle sizes on SOD activity of soiny mullet (*Liza haematocheila*) larvae (n=9). Data were presented as mean ± SD. Different letters indicate significant differences (P<0.05). Conversely, there is no significant difference between the same letters.

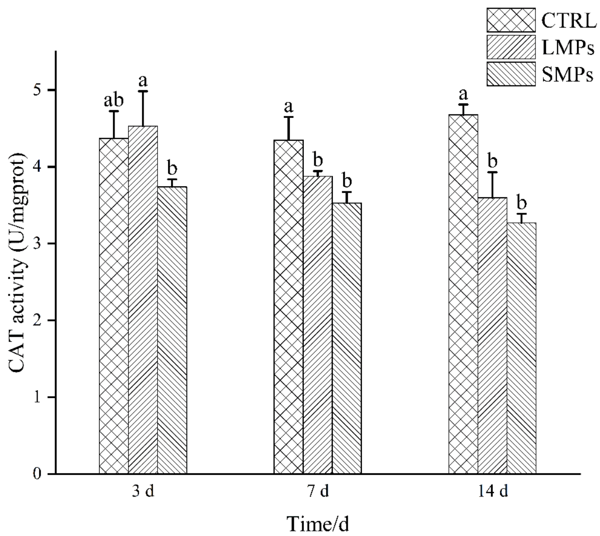

Figure 6. Effects of exposure to MPs with different particle sizes on CAT activity of soiny mullet (*Liza haematocheila*) larvae (n=9). Data were presented as mean ± SD. Different letters indicate significant differences (P<0.05). Conversely, there is no significant difference between the same letters.

Similarly, the MPs exposure increased the ALP activities of soiny mullet larvae throughout the trial period (Figure 8). At 3 dpe, the ALP activities in LMPs and SMPs were significantly higher than that of CTRL (P<0.05). At 7 dpe, the ALP activity in LMPs shared similar level as that in CTRL (P>0.05), while that in the SMPs was remained higher than that in the CTRL (P<0.05). At 14 dpe, the ALP activities in LMPs and SMPs were significantly higher than that in CTRL (P<0.05) (Figure 8).

Discussion

Effects of MPs on the Activities of Digestive Enzymes of Soiny Mullet Larvae

Digestive enzymes play important roles in the digestion and absorption of nutrients of animals (Ahmadifaret al., 2020). Various studies have shown that MPs challenge can negatively affect the activities of digestive enzymes of several fish species, such as orange-spotted grouper *(Epinephelus coioides* Hamilton, 1822) (Wang et al., 2022a), guppy (*Poecilia reticulata* reticulate W. Peters, 1859) (Huang et al., 2020), and large yellow croaker (*Larimichthys crocea* Richardson, 1846) (Gu et al., 2020). PPS and α-AMS are two important digestive enzymes involved in the digestion and absorption of proteins and carbohydrates. PPS is a digestive protease, secreted by the host cell, that functions to break down proteins in food into small peptide fragments. α-AMS is one of the extracellular enzymes that play an important role in the degradation of starch in living organisms. It acts on the a-1,4 glycosidic bond of the starch chain to break down starch into dextrins and reducing sugars. Our study found that MPs exposure inhibited the activities of PPS and α-AMS

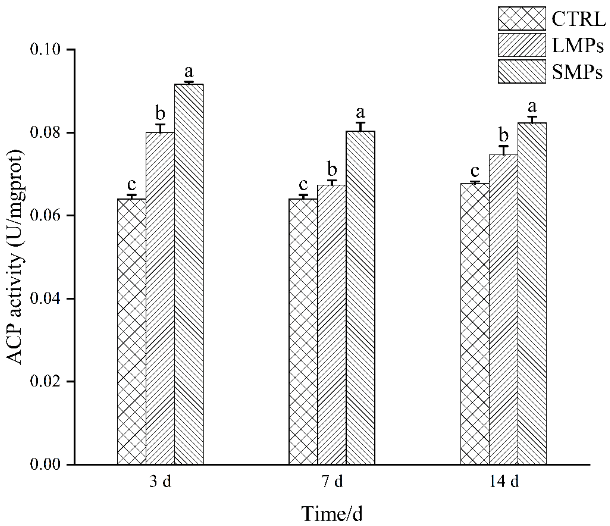

Figure 7. Effects of exposure to MPs with different particle sizes on ACP activity of soiny mullet (*Liza haematocheila*) larvae (n=9). Data were presented as mean ± SD. Different letters indicate significant differences (P<0.05). Conversely, there is no significant difference between the same letters.

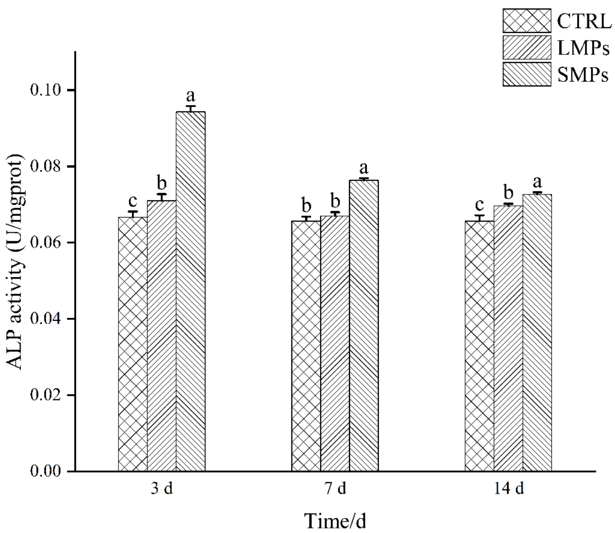

Figure 8. Effects of exposure to MPs with different particle sizes on AKP activity of soiny mullet (*Liza haematocheila*) larvae (n=9). Data were presented as mean ± SD. Different letters indicate significant differences (P<0.05). Conversely, there is no significant difference between the same letters.

in soiny mullet. Similar results were also observed in discus fish (*Symphysodon aequifasciatus* Pellegrin, 1904) after MPs (20µg/L; 200 µg/L) challenge for 28 days (Zhang et al., 2022). The reason for the activity inhibiting by the MPs exposure might be the accumulation of MPs in the gut, which causes gut blockage to produce a feeling of satiety and reduce food intake (Lu et al., 2016). When there were fewer proteins and carbohydrates in the food, there was less need for these enzymes that break down proteins and carbohydrates in the fish, which affected the activity of digestive enzymes. In addition, we found that the PPS activity was more strongly responsive to small MPs (0.5µm), while the α-AMS activity was more strongly responsive to large MPs (6µm). We speculated that MPs with different particle sizes could have various effects on different flora in the gut, which cause different microbiota dysbiosis and finally resulting in inconsistent levels of digestion and absorption of different nutrients by the body, and different digestive enzymes showing different activities, which was previously reported in the studies of Liu et al. (2019), Huang et al. (2020), Huang et al. (2022a) and Huang et al. (2022b).

Effects of MPs on the Activities of Antioxidative Enzymes of Soiny Mullet Larvae

The detoxification process in animals is the result of the joint action of multiple antioxidant defense enzymes. SOD and CAT are antioxidant enzymes involved in scavenging superoxide radicals. SOD firstly converts the O^{2-} into H_2O_2 to maintain the normal physiological activities of cells and the body and prevent excessive radicals from damaging macromolecules such as proteins, lipids, and DNA (Geracitano et al., 2002). Then, the CAT splits H_2O_2 into H_2O and O_2. When facing the stress of toxic substances in the environment, the body will induce an increasing activitiy of antioxidant enzymes to adapt to the environment change (Ajitha et al., 2021). Studies showed that 90-day exposure to food rich with MPs increased the SOD and CAT activities and induced oxidative stress in gilthead seabream (*Sparus aurata* Linnaeus, 1758) (Capó et al., 2021). In the present study, MPs exposure induced a significant reduction in the SOD and CAT activities of soiny mullet. Similarly, exposure to MPs (0.5 µm, 100µg/L; 0.5 µm, 1000µg/L; 5 µm, 100µg/L; 5 µm, 1000µg/L) for 21 days decreased the SOD and CAT activities and caused oxidative damage of loach (*Paramisgurnus dabryanus* Dabry de Thiersant, 1872) (Wang et al., 2022b). A study in common carp showed a reduction in SOD activity after an early rise in target organs, following MP exposure because ROS produced in tissues could not be removed instantly (Xia et al., 2020). However, Banaei (2022) found that exposure to MPs (175, 350, 700, 1400 µg/L) for 30 days, the activity of SOD was increased, while the activity of CAT was decreased, and oxidative stress was induced in common carp (*Cyprinus carpio* Linnaeus, 1758). High concentration and weak antioxidant capacity of soiny mullet larvae might be the reasons for these different results. In addition, we found that the SOD and CAT activities in SMPs were lower than those in LMPs, which might have been caused by the small particles of MPs (0.5 µm), which are more likely to accumulate in tissues and thus exhibit stronger cytotoxicity as previously explained by Choi & Hu (2008), Jeong et al. (2016) and Lu et al. (2016), leading to reactive oxygen species (ROS) production by NADPH oxidase, SOD converts superoxide radicals to hydrogen peroxide, and CAT converts hydrogen peroxide to water and oxygen as the prime protection mechanism against oxidative stress (Sahabuddin, et al., 2023).

Effects MPs on the Activities of Immune Enzymes of Soiny Mullet Larvae

Fish innate immunity is first defense line against pathogens or stress. ACP and ALP are important innate immune related enzymes (NavinChandran et al., 2014; Suvetha et al., 2015). ACP is a marker enzyme of cellular lysosomes and involves in the inflammatory response (Lallès, 2019; He et al., 2021), commonly found in macrophages and neutrophils, which are key components of the fish immune system. ALP is associated with damage to cell membranes, it is involved in a variety of physiological processes, including phosphate metabolism and transport. In the immune response, ALP can be involved in cell signalling and cell activation processes. ALP is also associated with damage to cell membranes (Banaee et al., 2019). Both ACP and ALP involve in the cellular phosphorylation and dephosphorylation, as well as reflecting the metabolic rate of toxic substances in the body (Ellis et al., 2011; Lu et al., 2019). Therefore, ACP and ALP are usually used as biomarkers of environmental pollution (Wen et al., 2018). In the present study, we found that MPs exposure significantly increased the activities of ACP and ALP in soiny mullet. Similar results were also observed in other animals. The study of Liu et al. (2019) revealed that 14 daysof MPs exposure (5 µm, 0.4 mg/L) significantly increased the activities of ACP and ALP and activated the immune system in Chinese mitten crab. Wen (2018) also found that MPs exposure (50µg/L; 500µg/L) for 14 days increased the activities of ACP and ALP and induced the immune responses in the tested fish larvae. Our result are in agreement with Pitt et al. (2018) and indicated that the innate immunity of animals is triggered to adapt to MPs in the environment, When fish were exposed to pathogens or inflammation, activation of the immune system increased the number of these immune cells, which may lead to elevated enzyme activity.

Conclusions

The effects of different size MPs on the activities of digestive enzymes, antioxidant enzymes and immune enzymes of soiny mullet larvae were investigated. To the best of our knowledge, this is the first report about the effect of MPs on the soiny mullet larvae. Our results demonstrated that the digestion and antioxidant system of soiny mulet larvae were inhibited by MPs exposures. And, the activities of immune enzymes were induced. Both sizes of MPs were toxic to soiny mullet larvae, and the small particles of MPs (0.5 µm) showed to be more toxic compared with the large particles of MPs (6 µm). These results provide solide basis for understanding the detriment of MPs in larvae fish. Notedly, the detoxification mechanism of microplastic on larvae fish needs further investigation.

Ethical Statement

Not applicable

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Author Contribution

Zhitao Qi: Supervision, Writing- Reviewing and Editing. Yingying Zhang; Shengyuan Zhang: Enzymes activities analysis. Yanming Sui: microplastics preparation and writing. Ke Sun; Qihuan Zhang: data analysis. Eakapol Wangkahart; Zisheng Wang: Supervision and writing.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

Not applicable

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