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**Artificial UVC-aging process aggravates the toxicity of
polystyrene microplastics to zebrafish, especially females**

Di Wu^{1, 2}, Zixuan Hu¹, Laura Carter², Paul Kay², Joseph Holden², Ying Yin^{1*},
Hongyan Guo¹

¹ State Key Laboratory of Water Pollution Control and Green Resource Recycling,

School of the Environment, Nanjing University, Nanjing, 210023, China

² water@leeds, School of Geography, University of Leeds, Leeds, LS2 9JT, UK

*Corresponding author. Tel.: +86-25-89680263; Fax: +86-25-89680263.

E-mail address: yinying@nju.edu.cn (Yin Y).

Abstract

The ecological risks of microplastics (MPs) are increasingly recognized. However, the role of the MP aging process on risks in freshwater ecosystems remains unknown. In this study, 500 nm polystyrene microplastics (PS-MPs) were subjected to artificial UVC (254 nm) irradiation for six months to induce accelerated photo-oxidation. Adult zebrafish were exposed to pristine and UVC-aged PS-MPs for 21 days to evaluate health related indicators. UVC photodegradation significantly altered the physicochemical properties of PS-MPs, including roughening of surfaces and generation of new oxygen-containing functional groups. Exposure to aged PS-MPs produced stronger biological responses than pristine particles, as indicated by integrated biomarker response (IBR) analysis, with elevated oxidative stress and changes in humoral immune indicators. Multi-omics analysis revealed that both pristine and aged PS-MPs induced gut microbiota dysbiosis and hepatic metabolic disorder, while the aged PS-MP exposure group showed broader metabolic perturbations in female zebrafish, including alterations in glycolipid and amino-acid metabolism pathways and enhanced oxidative stress responses. Correlation analysis indicated close associations among gut microbial shifts and metabolic alterations. Our results highlight the enhanced toxicity of aged PS-MPs on freshwater organisms and emphasize the importance of considering the aging process in toxicological assessments of MPs in freshwater systems.

Keywords: polystyrene microplastics, aging process, gut-liver axis, multi-omics analysis

1. Introduction

Microplastic (MP) pollution in freshwater ecosystems is attracting increasing concern. MPs can be ingested by various freshwater organisms directly, including fish, cladocerans and mussels, leading to adverse impacts [1, 2].

MPs can affect freshwater organism health at the individual, cellular, and molecular levels [3]. For example, the accumulation of MPs has been observed to block the digestive system, hindering nutrient absorption and damaging organism health [4]. MPs could induce oxidative stress, inflammation, DNA damage and immune inhibition [5-8]. Furthermore, there is the potential for ingested MPs to be transferred through the food chain and even to the human body, posing a threat to wider ecosystem stability and human health [9]. A comprehensive assessment of health effects and risks of MPs in freshwater ecosystems, accounting for potential changes in the properties and behavior of MPs during environmental exposure, is therefore necessary.

After being released into freshwater, MPs will undergo many biological and abiotic aging processes, such as via light, heat and oxidation, that can alter their properties and behavior [10]. Aging processes have the potential to affect the toxicity of MPs to freshwater organisms, but little is known about these processes. Previous studies have demonstrated that aged polyvinyl chloride (PVC) MPs showed greater toxicity on algae *Chlamydomonas reinhardtii* due to formation of carbonyl groups and increased zeta potential [11]. Michailidou et al. [12] found that aged polyethylene MPs (compared to pristine) caused greater health damage to freshwater fish *Perca fluviatilis* by inducing oxidative stress and affecting cellular health. Conversely, some studies have

demonstrated aging of MPs did not increase the toxicity to algae, daphnids and duckweed [13, 14]. Thus, it remains uncertain about the impacts of aging processes on the toxicity of MPs, and further study is needed. While solar radiation reaching freshwater systems is dominated by UVA and UVB wavelengths, accelerated UVC (254 nm) irradiation is frequently used in laboratory studies as a controlled photo-oxidation model to induce surface functionalization and carbonyl formation.

After exposure to MPs, ingestion is the major uptake pathway, however, MPs can also adhere to fish skin and transfer to other parts of the body [15, 16]. As an ideal model for toxicology and risk assessment, existing research has shown that MPs can cause damage to the health of zebrafish based on these interaction routes, including the gut-liver axis, metabolism, the nervous system, reproductive development, and humoral immune system [17-19]. Our previous study also demonstrated a range of sex-specific toxicity effects of polystyrene MPs (PS-MPs) on zebrafish [20]. Furthermore, there has been some research on the impacts of aged MPs on zebrafish larvae, indicating aggravated neurotoxicity, gut damage, lipid malabsorption, immune dysfunction and growth inhibition [21, 22]. However, there remains a need for research on the impacts of aged MPs on adult zebrafish health.

Accordingly, this study aims to investigate whether PS-MPs aging process influences their health impacts on adult female and male zebrafish over a 21-day exposure period. Specifically, we considered: (i) characterization of aged PS-MPs; (ii) the toxicity and mechanisms of influence for pristine and aged PS-MPs on zebrafish of both sexes. The toxicity on zebrafish health was evaluated by measuring humoral

indicators and oxidative stress. Multi-omics analysis was used to reveal the microbial and metabolic responses of zebrafish along gut-liver axis.

2. Materials and methods

2.1 PS-MPs preparation, aging treatment and characterization

Pristine PS-MPs (500 nm) were synthesized in the lab according to methods used in previous studies [23, 24]. The pristine PS-MPs were placed in a controlled aging chamber and subjected to continuous and uninterrupted UVC irradiation (254 nm; 4 × 20 W; surface intensity: 75 μW/cm²) for a total duration of 180 days. UVC irradiation represents a controlled laboratory aging method and was designed to accelerate photo-oxidative functionalization and carbonyl formation. After aging, particles were characterized by transmission electron microscope, Fourier Transform infrared spectroscopy (FTIR) and X-ray photoelectron spectroscopy (XPS). The carbonyl index (CI) was determined to characterize the aging degree [25].

$$CI = \frac{I_{1742}}{I_{1450}} \quad (1)$$

where I is the peak intensity.

2.2 Zebrafish culture and experimental setup

Zebrafish (5 months old) were supplied by the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China). The zebrafish were kept in ultraviolet sterilized and aerated water at 24 ± 1 °C with a 14:10 h light/dark cycle and were fed twice daily brine shrimp. After acclimation, zebrafish were randomly assigned to six experimental groups: female control group (F-control), female pristine PS-MPs treatment group (FP-PS-MP), female aged PS-MPs treatment group (FA-PS-MP), male control group (M-

control), male pristine PS-MPs treatment group (MP-PS-MP), male aged PS-MPs treatment group (MA-PS-MP). Each group contained four replicate glass tanks (n=4 tanks per group): 25 zebrafish were included in each tank. The exposure concentration (2.5 mg/L) used in this study is higher than most reported environmental concentrations. This elevated concentration was selected to serve as a controlled mechanistic stress-test, enabling the detection of biological responses and identification of sensitive endpoints under intensified exposure conditions, rather than to directly simulate environmental scenarios. Particles were kept in suspension through continuous gentle aeration. A 50% water change with fresh MP-spiked medium was performed every 48 hours to maintain concentration stability and water quality. After 21 days of exposure, zebrafish were anesthetized and sacrificed in ice water before obtaining the tissues.

2.3 Determination of Immunoglobulin M (IgM) in mucus, lipopolysaccharide (LPS) and triglyceride (TG) in blood serum and oxidative stress in liver

Skin mucus was collected according to methods outlined in a previous study [26] (Text S1). IgM content in mucus, LPS and TG level in zebrafish blood, reactive oxygen species (ROS) level, superoxide dismutase (SOD) activity and malondialdehyde (MDA) content in liver tissue homogenates (10 %) were measured by commercial kits (Jiancheng, Nanjing, China).

2.4 Integrated biomarker responses (IBR) analysis

Integrated biomarker response version 2 (IBRv2) analysis was conducted to summarize measured health-related indicator responses into one general index [27]. The health-related indicators included ROS, SOD, MDA, LPS, TG, and IgM. The

calculation formulae are listed in Text S2.

2.5 Gut microbiome analysis

The gut samples were collected, frozen in liquid nitrogen and then preserved at -80 °C. The details of DNA extraction, 16s rRNA gene sequencing, and data analysis are listed in Text S3.

2.6 Metabolomic analysis in the gut and the liver and correlation analysis

The gut and liver samples were collected, frozen in liquid nitrogen and then preserved at -80 °C for further analysis. The details of sample preparation and extraction, UPLC conditions, LC-MS/MS analysis, data analysis and interpretation and bioinformatics analysis are provided in Text S4. Correlation analysis (Pearson analysis and Mantel test) was performed to evaluate the relationship between gut microbiota and gut differentially expressed metabolites (DEMs), gut microbiota and liver DEMs, and gut DEMs and liver DEMs by the OmicStudio tools at <https://www.omicstudio.cn/tool> (R package *stats* and *ggcor*, $p < 0.05$, $\rho > 0.6$ or < -0.6).

2.7 Data analysis

The data are expressed as mean $\pm$ SD. One-way analysis of variance (ANOVA) with IBM SPSS Statistics 22 was used to evaluate the differences among different groups. $P < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1 Characterization of pristine and aged PS-MPs

The hydrodynamic diameter and PDI of pristine PS-MPs in exposure water were 584.3 ± 2.0 nm and 0.023 ± 0.021 , respectively, indicating relatively good dispersion

in the exposure medium. Smooth surfaces were observed in pristine PS-MPs while aged PS-MPs had rough surface and bulges (Fig. 1). PS-MPs was observed to change color from white to yellow after the UV aging process. XPS spectra showed that the O 1s proportion of aged PS-MPs increased from 8.03% to 19.92%, while the C 1s proportion of aged PS-MPs decreased from 91.97% to 80.08%. FTIR results (Fig. 1D) showed that new functional groups were found in the regions of 3100-3700 cm^{-1} , 1630-1840 cm^{-1} , 1340-1200 cm^{-1} , identified as -OH and C=O groups. The CI value of pristine and aged PS-MPs was 0.43 ± 0.01 and 3.08 ± 0.003 , respectively.

Several studies have demonstrated that laboratory accelerated oxidation of MPs could simulate the long-term aging process of MPs in the natural environment, and UV photodegradation can accelerate the aging process and alter physicochemical properties of MPs [28-30]. As with our study, Xiang et al. [31] also observed a wrinkled surface of aged PS-MPs; Chen et al. [32] found that UV radiation could change the color and increase O 1s proportion of PS-MPs, indicating that PS-MPs had been oxidized. The generation of new oxygen-containing functional groups from FTIR analysis further demonstrated this process had occurred. While surface free radical content was not directly quantified, their role in the degradation process is strongly inferred from these oxygen-containing transformations, which are the formation of peroxy radicals and hydroperoxide intermediates, resulting in the peroxy radicals undergoing chain scission to form carbonyl compounds [33]. Combined, our results show that UV photodegradation altered the physicochemical properties of PS-MPs.

3.2 Humoral indicators, oxidative stress and IBR analysis

Humoral indicators were determined in blood serum and skin mucus. As shown in Fig. 2A, both pristine and aged PS-MPs significantly increased IgM level in females. The increase of IgM level in the FA-PS-MP group is greater than that in the FP-PS-MP group. The skin mucus of fish is a barrier against various chemical, physical and biological stressors, regarded as the first line of defense against infection [34]. As one of the immune parameters in mucus, IgM serves as an immune effector in the humoral immune system of fish [35]. To our knowledge, there is little research focusing on the effects of MPs on zebrafish mucus immunity. Yang et al. [36] showed that PS-MPs had no effect on IgM level in larval zebrafish. However, our study on adult zebrafish found that IgM level increased in female mucus in both PS-MP groups, and the FA-PS-MP group showed a greater increase in mucus compared to the FP-PS-MP group, indicating PS-MPs might induce immunostimulatory effects on female zebrafish and aged PS-MPs induced more of these effects.

Fig. 2B and C show that aged PS-MPs raised LPS and TG content significantly in both sexes. However, no differences were found between controls and pristine PS-MP groups. Aged PS-MPs increased ROS level and SOD activity in females, induced SOD activity and MDA content in males, while there was no change between controls and pristine PS-MPs treatments (Fig. 2D-F).

The release of LPS is related to changes in the composition of gut microbiota [37]. Luan et al. [38] similarly found that polyglycolic acid MPs could induce gut microbiota dysbiosis in zebrafish, leading to the overproduction of LPS and adverse effects on liver health by the gut-liver-brain axis. In our study, the increased relative abundance of

190 gram-negative bacteria and upregulation of LPS biosynthesis could increase LPS level
191 in the gut (section 3.3.1), potentially inducing gut inflammatory responses [39]. The
192 increased serum LPS level in aged PS-MP groups suggested a compromised integrity
193 of the gut barrier, leading to the discharge of gut LPS to the bloodstream [40], which
194 may further induce immune and inflammatory responses and oxidative stress in the
195 liver [41, 42].

196 Oxidative damage is one of the major toxicity mechanisms of MPs to zebrafish
197 [43]. In our study, aged PS-MPs induced oxidative stress and lipid peroxidation in the
198 zebrafish liver, which may be explained by their changed surface characteristics,
199 including the increase of oxygen-containing functional groups and free radicals [44].
200 The oxidative imbalance could damage hepatic cells and disrupt the homeostasis of
201 liver lipid metabolism [45, 46]. As a widely present lipid compound in organisms and
202 one of the most important forms of energy storage, the primary site of TG synthesis is
203 the liver [47]. The increase in serum TG levels indicated an increase in liver lipid levels
204 and their release into the systemic circulation. The accumulation of TG in the liver will
205 induce liver dysfunction and damage to liver cells, leading to liver lipid metabolism
206 disorders [48], and therefore the increased content of TG, ROS, SOD and MDA
207 observed in our study in response to aged MP exposure suggests lipid metabolism
208 dysbiosis and oxidative damage occurred in the liver.

209 The IBR analysis integrated multi-indicator responses (Fig. 2G and H). The value
210 of the IBRv2 index in the aged PS-MP treatment group was higher than that in the
211 pristine PS-MPs treatment in both sexes. Specifically, the values increased from 6.38

(FP-PS-MP) to 11.15 (FA-PS-MP) in females, and from 6.72 (MP-PS-MP) to 12.10 (MA-PS-MP) in males (Table S1). A high IBR value usually indicates an increase in toxic stress in the test organism [49]. The higher IBR value in the aged PS-MP treatment groups further demonstrated that UVC-aged PS-MPs showed larger biomarker perturbations to zebrafish than pristine PS-MPs under these experimental conditions.

3.3 The adverse health impact on zebrafish induced by pristine and aged PS-MPs through the gut-liver axis

By inducing oxidative stress and the disruption of humoral indicators in zebrafish, aged PS-MPs induced more adverse effects than pristine PS-MPs. To clarify the impact of PS-MPs on zebrafish health and the toxicity differences between pristine and aged PS-MPs, it is necessary to further analyze molecular processes along gut-liver axis to explain the changes in gut microbiome and metabolism.

3.3.1 Gut microbiota composition and predicted function

The Venn diagram (Fig. S1 A and B) showed that PS-MPs changed the number of OTUs of gut microbiota compared to the control group. To quantify the overall community shift, beta-diversity was assessed using UniFrac distances. Aged PS-MP exposure induced a significant shift in the female intestinal community (Unweighted UniFrac, $p = 0.033$), whereas the male group showed no such significant divergence ($p > 0.05$) (Fig S1E and F). Consistent results were found by PLS-DA analysis (Fig. S1C and D).

The relative abundance of gut microbiota composition in the control, pristine and aged PS-MP treatment groups was exhibited at the phylum level in Fig 3A, 3B, S2A

and S2B. Firmicutes and Bacteroidota increased while Proteobacteria and Actinobacteriota decreased after exposure to pristine PS-MPs. Aged PS-MPs increased Proteobacteria and Bacteroidota and reduced Firmicutes and Actinobacteriota. In male zebrafish, pristine and aged PS-MPs both increased Firmicutes, Actinobacteriota and Bacteroidota while decreasing Proteobacteria.

As Proteobacteria are often associated with gut inflammation [39], our results showed an upward trend in their relative abundance specifically in female zebrafish following exposure to aged PS-MPs. Although this enrichment did not reach statistical significance after FDR correction, this compositional shift is noteworthy, as microbial dysbiosis in the gut is frequently linked to increased gut permeability and the subsequent translocation of bacterial endotoxins, such as LPS into the bloodstream [40, 50]. This exploratory finding is consistent with the significantly elevated serum LPS levels and oxidative stress biomarkers observed in the female FA-PS-MP group (Section 3.2), suggesting that aged particles may trigger a more pronounced inflammatory response via the gut-liver axis in females. The sex-dependent increase of Proteobacteria may also be related to physiological differences between male and female fish. Sex-dependent host-microbiome interactions have been reported in zebrafish and other aquatic organisms, where differences in metabolism, immune regulation, and endocrine signaling can influence microbial responses to environmental stressors [51-53]. In addition, the gut microbiota plays an important role in regulating host metabolism and immune responses in zebrafish, and disturbances in microbial composition can influence metabolic homeostasis [54, 55]. Therefore, physiological

differences between males and females may create distinct intestinal microenvironments that influence microbial susceptibility to MPs and may create gut conditions that preferentially favor the proliferation of opportunistic Proteobacteria in females. Nevertheless, the precise mechanisms underlying this sex-dependent microbial response require further investigation. At the genus level, the FA-PS-MP group exhibited an upward trend in the relative abundance of *Pseudomonas* and *Aeromonas* (Fig. S2C). Although these changes did not achieve statistical significance after FDR correction, their increased presence is consistent with the overall expansion of the Proteobacteria phylum observed in females exposed to aged particles. These genera are fish pathogens and can cause several fish diseases, such as lung infections, enteritis, and ulcer disease [56, 57]. Their elevated levels suggest a stressed gut environment, which is consistent with the elevated LPS levels and oxidative stress biomarkers in the female FA-PS-MP (Fig. 2B).

The Firmicutes are associated with immune function and digestive health and the Bacteroidetes are associated with the digestion of cellulose and monounsaturated fatty acids in fish [58, 59]. In contrast to several studies [60-62], Firmicutes and Bacteroidetes increased and Proteobacteria reduced in the FP-PS-MP, MP-PS-MP, and MA-PS-MP groups compared to controls. Gu et al. [63] found a similar outcome in the gut of large yellow croaker *Larimichthys crocea* after PS nanoplastics exposure, and suggested that an increase in gut beneficial bacteria would help the species to cope with the growth of potential pathogenic bacteria. Considering the complexity and interactions of gut microbiota [64], our results highlight the potential for dysbiosis in

the gut microbiota by PS-MPs, which may further result in oxidative stress and inflammatory responses.

As the FA-PS-MP group exhibited the most distinct alterations in both microbial composition and liver metabolism (section 3.3.4), predicted functions of gut microbiota in the FA-PS-MP group was explored by PICRUSt2. Several pathways exhibited significant upregulations compared to the F-control group, including energy metabolism, membrane transport, metabolism of cofactors and vitamins, and glycan biosynthesis and metabolism (Fig. S2E).

We found that aged PS-MPs could affect element metabolism (C, N, S, and P) of female gut microbiota. The phosphotransferase system is related to the transport and phosphorylation of carbohydrates [65]. Several studies also demonstrated the potential modification of microbial element metabolism functions induced by MPs, including C/N/S/P metabolism [66, 67]. In addition, upregulation of LPS biosynthesis of gut microbiota paralleled an increased level of LPS in the blood. The increase of LPS level could induce gut inflammation by activation of innate immunity and promote pro-inflammatory gene expression [68]. Thus, the upregulation of LPS biosynthesis may indicate a more invasive gut microbiota.

3.3.2 Gut metabolomic analysis and KEGG pathway analysis

While no metabolites passed the stringent False Discovery Rate (FDR) correction ($q < 0.05$), an exploratory analysis based on nominal p-values ($p < 0.05$) revealed a distinct metabolic signature in the FA-PS-MP group. Although Principal Component Analysis (PCA) showed that the FA-PS-MP group exhibited a more pronounced

300 separation from the control compared to the FP-PS-MP group (Fig. S3A), these shifts
301 represent exploratory trends rather than statistically significant deviations. In the FA-
302 PS-MP group, 129 DEMs were identified (nominal $p < 0.05$), with a majority being up-
303 regulated. In contrast, fewer DEMs were observed in the FP-PS-MP group (Fig. S3B
304 and C). Hierarchical clustering (Fig. S4) further indicated that the aged PS-MPs exerted
305 a broader influence on the metabolome than pristine particles, particularly affecting
306 lipids, nucleosides, and organic acids.

307 Functional enrichment analysis revealed that aged PS-MPs induced a broader and
308 more complex metabolic disruption in female zebrafish compared to pristine PS-MPs.
309 As shown in the KEGG category overview (Fig. 3C and D), the FA-PS-MP group
310 exhibited perturbations across a higher number of metabolic categories, particularly
311 within nucleotide metabolism and human disease-related pathways, which were less
312 prominent in the FP-PS-MP group. As visualized in the enrichment bubble plots (Fig.
313 S3D and E), the FA-PS-MP group was associated with a wider array of metabolic
314 perturbations. Notably, glycerophospholipid and ether lipid metabolism emerged as the
315 enriched pathways in the FA-PS-MP group. Conversely, the metabolic influence of the
316 FP-PS-MP group was more restricted, primarily centering on the PPAR signaling
317 pathway and arachidonic acid metabolism. These results suggest that UVC aging
318 process significantly expands the metabolic footprint of PS-MPs, intensifying the strain
319 on hepatic lipid and nucleotide homeostasis.

320 Based on the KEGG enrichment analysis (Fig. S3D and E), the lipid metabolism
321 category emerged as a prominent metabolic target in the FA-PS-MP group, including

glycerophospholipid metabolism and ether lipid metabolism. As one of the fundamental gut functions, lipid metabolism is important for energy production and metabolism [39]. Similarly, several studies have shown that MPs can induce disruptions in gut lipid metabolism in fish, causing adverse health impacts [5, 69]. Rossi et al. [70] demonstrated that PS nanoplastics could readily permeate into lipid membranes, altering their structure. Cedervall et al. [71] found that PS nanoplastics can bind to apolipoprotein A-I in fish to interfere with lipid metabolism.

3.3.3 Correlation between influenced gut microbiota and gut DEMs in female zebrafish

Pearson correlation analysis was conducted to evaluate the association between gut microbiota and gut DEMs. In the FP-PS-MP group, only Actinobacteriota was related to 8-hydroxy-5Z, 9E, 11Z, 14Z-eicosatetraenoic acid and 17-aminogeldanamycin (Fig. S5). In the FA-PS-MP group, gut microbiota was closely related to lipid metabolites. Specifically, Proteobacteria was closely related to larsucosterol, delta-24-cholesterol, Sphingomyelin (SM) (d16:2(4E, 8Z)/PGJ2), and Phosphatidylethanolamine (PE) (18:2/0:0). Bacteroidota was significantly associated with glycerophosphocholine and larsucosterol. These results indicate the potential association between gut microbial alteration and gut lipid metabolism.

3.3.4 Hepatic metabolomic alterations induced by pristine and aged PS-MPs and KEGG pathway analysis

Volcano plots (Fig. 4) highlight several candidate metabolites with substantial effect sizes, particularly within the glycerophospholipid category. In the female FA-PS-MP group, 95 DEMs were identified, with a predominant trend toward up-regulation

(70 up-regulated and 25 down-regulated). In contrast, the FP-PS-MP group exhibited a much more restricted metabolic response, with only 27 DEMs (8 up-regulated and 19 down-regulated). Regarding the male groups, exposure to pristine PS-MPs resulted in 67 DEMs (30 up-regulated and 37 down-regulated), while the MA-PS-MP group showed 64 altered metabolites (24 up-regulated and 40 down-regulated). Hierarchical clustering (Fig. S7) further corroborated that aged PS-MPs induced a more extensive redistribution of the metabolome in females than in males.

Among all treatment groups, the FA-PS-MP group displayed the highest number of metabolic perturbations, suggesting a distinct sex-specific sensitivity and a more profound impact of aged particles on hepatic homeostasis compared to pristine PS-MPs. In addition, most of the DEMs in the FA-PS-MP group and the MA-PS-MP group belonged to lipids, including glycerophospholipids, fatty acyls, steroids and steroid derivatives and prenol lipids (Fig. S9). Glycerophospholipids are the main components of biological membranes and precursors to lipid mediators, associated with numerous metabolic diseases [72]; fatty acyls are important sources of energy and play a pivotal role in lipid composition [73]; and steroid hormones play a regulatory role in regulating lipid metabolism and inflammation. [74]. The changes in these metabolites suggested potential liver damage and dysregulation in lipid metabolism induced by aged PS-MPs. Impacts on hepatic lipid metabolism were previously demonstrated as a component of hepatotoxicity of MPs [38, 72, 75]. The disruption of lipid metabolism can be induced by oxidative stress [76], which was observed in the aged PS-MP treatment groups in our study (Fig. 2). Thus, the metabolome analysis provides further evidence that

exposure to aged PS-MPs could result in metabolic disorders, particularly affecting lipid metabolism.

In the female groups, pristine PS-MPs altered carbohydrate metabolism and amino acid metabolism; while aged PS-MPs altered more pathways, including membrane transport, digestive system, carbohydrate metabolism, endocrine system, amino acid metabolism, metabolism of cofactors and vitamins, and signal transduction (Fig. 4 and S10). In the male groups, pristine and aged PS-MPs both altered global and overview maps, biosynthesis of other secondary metabolites, amino acid metabolism, and nucleotide metabolism.

MPs were demonstrated to disrupt carbohydrate and amino acid metabolism in fish, leading to energy insufficiency and disorder which is a finding consistent with previous research [77-79]. Specifically, our results showed that pristine and aged PS-MPs both influenced amino acids metabolism and carbohydrate metabolism in both sexes, which are basic biological processes for maintaining liver function and overall metabolic homeostasis [80, 81]. Among differential amino acids, tryptophan and phenylalanine are related to immunity and stress response [82]. Choi et al. [83] also found the exposure to polyethylene terephthalate MPs affected tryptophan and phenylalanine metabolism, accompanied by inflammatory response and oxidative stress in juvenile rockfish. The disruptions in these amino acid metabolism pathways can lead to potential damage to protein synthesis and immunity.

In addition, there were some uniquely influenced metabolic pathways (e.g. membrane transport, digestive system, endocrine system) in the FA-PS-MP group

compared to the F-control and the MA-PS-MP group, indicating that exposure to pristine and aged PS-MPs may disrupt different basic metabolic mechanisms as well as sex-specific impacts of aged PS-MPs. In our study, betaine, enriched in the ABC transporter pathway, was down-regulated in the FA-PS-MP group. This indicated that aged PS-MPs might disrupt the balance of the substrate transport system and affect energy metabolism [84], which is consistent with a previous study [85]. Meanwhile, betaine has strong antioxidant properties and plays an important role in resisting oxidative damage [86]. The significant decrease of betaine level indicated its contribution to defend against oxidative damage and repair ROS-induced damaged membranes. Bile secretion and pancreatic secretion are involved in the digestive process, and the secreted bile acids, hormone and digestive enzymes can regulate lipid and glucose metabolism, contributing to immunity and glucose homeostasis [87, 88]. Thus, the effects of aged PS-MPs on the digestive system indicated potential gut function disorder in female zebrafish.

3.3.5 Correlation between influenced gut microbiota and liver DEMs

The correlation analysis revealed the complicated association between gut microbiota and hepatic DEMs. As shown in Fig. 5A, in the FP-PS-MP group, Bacteroidota had a significant positive correlation with glycerophosphoserine (GPSer) (22:6/18:0), and Actinobacteriota had a significant positive correlation with GPSer (22:6/18:0) and glycerophosphocholine (GPCho) (17:1/17:1), which belong to glycerophospholipids. In the FA-PS-MP group, the associations were more complex. Proteobacteria, Firmicutes and Bacteroidota were positively correlated with lipids,

amino acids and carbohydrates metabolites (Fig. 5B). Among them, lipid-related metabolites accounted for the majority, including glycerophospholipids, fatty acyls, steroids and steroids derivatives, and prenol lipids. Meanwhile, the Pearson analysis showed that the DEMs were interrelated with each other. In the MP-PS-MP group (Fig. S11A), only Bacteroidota had a significant positive correlation with organooxygen compounds and lipids metabolites, while Proteobacteria was positively correlated with lipids metabolites and negatively correlated with D-valine in the MA-PS-MP group (Fig. S11B).

Changes in gut microbiota can impact the gut barrier function, leading to the transmission of endogenous and exogenous substances produced by microbiota to the liver through the gut-liver axis, affecting zebrafish health [89]. The gut-liver axis provides a conceptual framework for understanding interactions between intestinal microbiota and host metabolism. In this study, the observed correlations between gut microbiota and liver metabolites suggest coordinated responses between gut microbial composition and hepatic metabolic profiles following PS-MPs exposure. Previous studies have also reported associations between microplastic exposure, microbiota dysbiosis, and metabolic disturbances [90, 91], which is consistent with our findings. Notably, compared to the FP-PS-MP group, the FA-PS-MP group exhibited more complex and extensive associations, particularly involving lipid metabolites such as glycerophospholipids (e.g., GPSer, GPCho, and Phosphatidylcholine (PC)), as well as amino acids and carbohydrates. These findings suggest the associations between microbial variation and alterations in hepatic glycolipid metabolism in females exposed

to aged PS-MPs. However, these correlation-based results may be inherently associative and the metabolomic results are derived from untargeted profiling and therefore require further biological validation. Future studies should include targeted quantitative validation of representative metabolites (e.g., lipid-related metabolites) as well as functional experiments to clarify the potential links between gut microbiota dynamics and hepatic metabolic alterations. Approaches such as tracer-based metabolite tracking, intestinal permeability assays, LPS neutralization experiments, or germ-free/antibiotic-mediated microbiome modulation would help further elucidate these relationships.

3.3.6 Correlation between influenced gut and liver lipid metabolites in the FA-PS-MP group and potential toxicity mechanism of aged PS-MPs on female zebrafish

Based on the results from gut and liver metabolomics, lipid metabolism was considered as the significantly influenced pathway in both the gut and liver metabolome. Meanwhile, gut microbiota was closely related to multiple lipid metabolites in both the gut and the liver. A correlation network analysis was conducted to identify the association and metabolic interactions across the two tissues, including 9 lipid metabolites from the gut and 12 lipid metabolites from the liver. Lipid metabolites in the gut were closely associated with lipid metabolites in the liver (Fig. 6A).

Glycerophospholipids products (PC, GPSer, GPCho in the liver; PE, glycerophosphocholine, glyceryl phosphorylcholine in the gut) appeared to weigh high in the correlation network, indicating glycerophospholipid metabolism disorder and the interplay between the two tissues. While pristine MPs have been documented to disturb

general lipid homeostasis in zebrafish [92], our findings reveal that mechanical aging potentially intensifies this disruption.

Because aged PS-MPs induced greater negative impact on gut microbiota composition and metabolic profiles in female zebrafish, further attention was given to the potential biological responses associated with aged PS-MPs exposure in female zebrafish. Based on the combined observations from microbiome profiling, metabolomic analysis, and oxidative stress biomarkers, aged PS-MPs exposure was associated with shifts in gut microbial composition, alterations in glycolipid- and amino acid-related metabolic pathways, and increased oxidative stress (Fig. 6B). These patterns suggest a possible interaction between gut microbiota dynamics, host metabolic responses, and oxidative stress under aged PS-MPs exposure. However, these relationships are based on associative evidence and should be interpreted cautiously, as additional functional and mechanistic experiments are required to confirm the underlying toxicity pathways.

3.4 Limitations and future work

This study provides novel insights into the toxicity mechanisms of pristine and aged PS-MPs on zebrafish health. However, there are still some aspects that require further investigation to comprehensively understand the toxicological impacts and potential risks of pristine and aged MPs to freshwater ecosystems. In-depth research should be conducted on the physicochemical property changes of aged MPs to better understand the mechanism of the toxicity changes. Targeted metabolite quantification and functional experiments are needed to validate the proposed pathways and clarify

the mechanistic links between gut microbiota and host metabolism under PS-MPs exposure. Moreover, future studies should incorporate environmentally relevant aging processes (e.g., UVA/UVB exposure, mechanical abrasion) and lower, field-realistic concentrations to better assess ecological relevance and refine risk assessment. Finally, future studies could investigate the toxicity of MPs on freshwater-sediment ecosystem instead of individual species, studying the interactions between plants, animals, water and sediment.

4. Conclusions

This study revealed that aged PS-MPs exerted more adverse effects than pristine PS-MPs, causing a stronger negative impact on zebrafish health. Exposure to aged PS-MPs increased the IgM, LPS and TG level and induced oxidative stress in zebrafish. The potential toxicity mechanism could be driven by the changed physicochemical properties of aged MPs, including the increase of oxygen-containing functional groups and free radicals. Meanwhile, female zebrafish showed greater changes in gut microbiota composition and metabolic profiles than males, suggesting potential sex-dependent responses to aged PS-MPs exposure. Both pristine and aged PS-MPs were associated with shifts in gut microbial composition and alterations in hepatic metabolic profiles. In particular, aged PS-MP exposure were accompanied by increased relative abundance of Proteobacteria in female zebrafish and broader changes in metabolites related to lipid, amino acid, and carbohydrate metabolism. Correlation analyses further indicated coordinated patterns between microbial composition and host metabolic responses in the gut and liver. Together, these results suggest that aged PS-MPs may

influence host physiology through combined changes in microbial composition, metabolic responses, and oxidative stress. Overall, our findings provide new insights into the potential health implications of aged MPs in freshwater organisms.

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Figure captions

Figure 1. Characterization of pristine and aged PS-MPs. TEM of PS-MPs after UV aging process. A, pristine PS-MPs; B, aged PS-MPs. XPS (C) and FTIR (D) spectra of pristine and aged PS-MPs.

Figure 2. IgM level (A), LPS level (B), TG level (C), ROS level (D), SOD activity (E), MDA content (F), and IBRv2 analysis (G, female zebrafish; H, male zebrafish) of zebrafish after exposure to pristine and aged PS-MPs for 21 days. Data represent mean $\pm$ SD. Different letters indicate significant differences between treatments ($p < 0.05$), the lowercase letters represent significant difference in female groups and uppercase letters represent those in male group.

Figure 3. Gut microbiota composition at phylum level among different treatment groups after exposure to pristine and aged PS-MPs for 21 days. A, female zebrafish; B, male zebrafish. KEGG pathway annotation in female zebrafish gut after exposure to pristine and aged PS-MPs for 21 days. C, FP-PS-MP group; D, FA-PS-MP group.

Figure 4. Volcano plot (A-D) and KEGG pathway enrichment (E and F) in zebrafish liver after exposure to pristine and aged PS-MPs for 21 days. A, MP-PS-MP group; B, MA-PS-MP group; C, FP-PS-MP group; D, FA-PS-MP group; E, FP-PS-MP group; F, FA-PS-MP group.

Figure 5. Correlation between gut microbiota and liver DEMs after exposure to pristine and aged PS-MPs for 21 days. A, FP-PS-MP group; B, FA-PS-MP group.

Figure 6. A, Relevance network between gut lipid metabolites and liver lipid metabolites in the FA-PS-MP group. B, a conceptual model of aged PS-MPs affecting

816 the health of female zebrafish through the gut-liver axis.

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