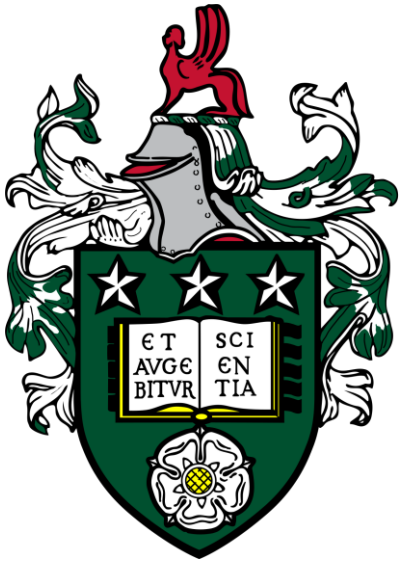


Ecotoxicological impacts of polystyrene micro- and nanoplastics in freshwater systems: gender-specific, aging-driven, and sediment-associated effects

Di Wu

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This thesis includes material published in peer-reviewed journals, in accordance with the University of Leeds regulations for doctoral theses.

The thesis comprises an overall introduction, followed by three research chapters based on published manuscripts. These chapters are integrated through a discussion synthesis, and the thesis concludes with limitations, future research directions and an overall conclusion. The structure is designed to ensure intellectual coherence and to demonstrate the original contribution of the candidate across all chapters.

Author contribution statement

I confirm that the work submitted is my own, except where work that has formed part of jointly authored publications has been included. I confirm that appropriate credit has been given within the thesis where reference has been made to the work of others.

Jointly authored publications used in the thesis:

(1) The work in Chapter 2 of this thesis has been published as follows:

Wu, D., Carter, L., Kay, P., Holden, J., Yin, Y., & Guo, H. (2025). Female zebrafish are more affected than males under polystyrene microplastics exposure. *Journal of Hazardous Materials*, 482, 136616. DOI: 10.1016/j.jhazmat.2024.136616.

(2) The work in Chapter 3 of this thesis has been published as follows:

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Abstract

Micro- and nanoplastic pollution is an increasingly pervasive problem in freshwater ecosystems, yet uncertainties remain regarding how particle transformation, biological sex, and cross-compartment interactions influence biological responses and ecosystem functional processes. This thesis investigates the biological effects of polystyrene (PS) micro- and nanoplastics in freshwater systems using a multi-scale laboratory framework. PS was selected for its widespread use in microplastic ecotoxicology, environmental occurrence, and suitability for investigating controlled particle aging and biological responses. A multi-scale approach was adopted to examine responses across interconnected biological and sediment-associated levels.

Zebrafish (*Danio rerio*) exposures examined responses to pristine and UV-aged PS particles, focusing on gender-specific responses, oxidative stress, metabolism, gut microbiota, and reproduction. A freshwater-sediment microcosm incorporating submerged plants (*Vallisneria spiralis*), freshwater snails (*Lymnaea stagnalis*), and artificial sediment assessed organismal stress and ecosystem functional responses.

The results demonstrate that: (1) 1 μm pristine PS microplastics induce oxidative stress, gut microbiota disturbance and liver metabolic alteration in zebrafish, with females showing broader physiological and metabolic responses than males. Altered sex hormone levels and reduced egg production in females indicated potential reproduction-related adverse outcomes; (2) UV aging modifies properties of 500 nm PS nanoplastics and was associated with stronger stress-related responses than pristine particles, including oxidative stress, gut microbiota dysbiosis, and metabolic disturbance related to gut-liver axis, particularly in females; (3) mechanically weathered PS microplastics induced

stronger stress-related responses in *V. spiralis* and *L. stagnalis* than pristine particles, while both pristine and mechanically weathered PS microplastics increased the ecosystem multifunctionality. This increase was mainly driven by increased dissolved organic carbon, microbial respiration and enzymatic activity, indicating short-term sediment-associated functional stimulation rather than improved ecosystem health or long-term resilience. Overall, this thesis highlights the importance of integrating ultraviolet aging, mechanical weathering, biological sex and cross-scale processes to improve freshwater PS micro- and nanoplastic risk assessment.

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Abbreviations

AChE	acetylcholinesterase
AKP	Alkaline phosphatase
ANOVA	Analysis of variance
BG	β-1,4-glucosidase
CAT	Catalase
Chl-a	Chlorophyll-a
Chl-b	Chlorophyll-b
CI	Carbonyl index
CP	Cellophane
DEMs	Differentially expressed metabolites
DOC	Dissolved organic carbon
DW	Dry weight
E2	17β-oestradiol
EMF	Ecosystem multifunctionality
FA	Female aged polystyrene nanoplastics treatment group
F-control	Female control group
FP	Female pristine polystyrene nanoplastics treatment group
FTIR	Fourier transform infrared spectroscopy
H&E	Haematoxylin and eosin
HPGL axis	Hypothalamus-pituitary-gonadal-liver axis
IBR	Integrated biomarker response
IgM	Immunoglobulin M
IRMS	Isotope ratio mass spectrometry
LPS	Lipopolysaccharide

LZM	Lysozyme
MA	Male aged polystyrene nanoplastics treatment group
M-control	Male control group
MDA	Malondialdehyde
MP	Male pristine polystyrene nanoplastics treatment group
PA	Polyamide
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PCoA	Principal coordinate analysis
PE	Polyethylene
PET	Polyethylene terephthalate
PLA	Polylactic acid
PP	Polypropylene
PS	Polystyrene
PTFE	Polytetrafluoroethylene
PU	Polyurethane
PVC	Polyvinyl chloride
PVF	Polyvinyl fluoride
ROS	Reactive oxygen species
SEM	Scanning electron microscopy
SOD	Superoxide dismutase
T	Testosterone
T-AOC	Total antioxidant capacity
TC	Total carbon
TG	Triglyceride

TIC	Total inorganic carbon
TN	Total nitrogen
TOC	Total organic carbon
UV	Ultraviolet
VIP	Variable importance projection
WWTPs	Wastewater treatment plants
XPS	X-ray photoelectron spectroscopy

Chapter 1. Polystyrene (PS) micro- and nanoplastics in freshwater ecosystems: Definitions, sources, transformation, biological responses, and knowledge gaps

1.1 General introduction

Plastics have become integral to modern society due to their durability, versatility, and low production cost and their global production has increased dramatically over the past seven decades (Zhao et al., 2024a). Due to their extensive production, diverse applications, and persistent nature, plastic wastes are continuously introduced into aquatic environments, where they have become a major source of environmental pollution (Zhang et al., 2021; Horton, 2022). Once released into the environment, plastic wastes undergo progressive fragmentation through physical, chemical and biological processes (Yang et al., 2024). These fragmentation pathways ultimately give rise to smaller plastic debris, termed microplastics, and even smaller nanoplastics, which have emerged as contaminants of global concern due to their persistence, ubiquity, and potential to cause harm across multiple levels of biological organization (Morales-Espinoza et al., 2025).

The ubiquitous presence of microplastics in aquatic ecosystems and their potential ecological risks have been widely reported (Galloway & Lewis, 2016; Koelmans et al., 2022; Vethaak & Legler, 2021). Freshwater systems are of particular concern, as they serve not only as essential drinking water sources but also as the major route for land-based microplastics entering marine environments (Kye et al., 2023). Although microplastic research initially focused on marine systems, freshwater environments are increasingly recognized as complex, dynamic, and ecologically rich habitats with unique physicochemical characteristics that influence microplastic behaviour and bioavailability (Lambert & Wagner, 2018). Many existing studies have viewed freshwater systems as transport pathways to marine environments, overlooking their ecological importance and close connections to humans (Zhao et al., 2024a). Thus, further investigation into the

effects of microplastics in freshwater ecosystems is urgently needed.

The overarching aim of this thesis is to investigate the biological effects and potential toxicity-related adverse outcomes of polystyrene (PS) micro- and nanoplastics in freshwater organisms and ecosystem processes. The rest of this chapter provides a comprehensive overview of microplastic pollution in freshwater systems and summarizes the physicochemical and biological impacts of microplastics on freshwater ecosystem, with a particular focus on PS micro- and nanoplastics. This chapter discusses the research status of micro- and nanoplastic effects on freshwater biota, current knowledge gaps and further research directions. The development of the proceeding chapters of research is based on these knowledge gaps and research directions.

1.2 Definitions and sources of micro- and nanoplastics

Microplastics are typically defined as plastic particles ranging from 1 μm to 5 mm with variable shapes; and nanoplastics are defined as plastic particles between 1 nm and 1 μm (Ali et al., 2024; Sangkham et al., 2022). They are generally classified into primary and secondary particles (Wagner et al., 2014).

Primary microplastics are manufactured to be of a microscopic size for specific industrial or personal applications, mainly including personal care products, industrial abrasives, paint, road marking and textiles (Upadhyay et al., 2024). As shown in Fig. 1.1, the generation of primary microplastics is closely linked to various sectors. Microplastics can be generated during plastic production and processing, either as intentional additives or as byproducts of industrial activities (El-Sherif et al., 2022). Various plastic manufacturing processes, including moulding, extrusion, and cutting, produce plastic wastes that may subsequently fragment into microplastics over time (Sheriff et al., 2023). Commercial activities, such as car and clothing sales, further contribute to microplastic generation (Ali et al., 2024; Nayal & Suthar, 2022). Domestic sources are primarily associated with the use and handling of daily consumer products, including the release of microbeads, which are small plastic particles intentionally added to certain personal care and cleaning products (Nayal & Suthar, 2022). Collectively, these manufacturing, commercial, and domestic processes result in the continuous release of microplastics into

freshwater systems.

Secondary microplastics are generated through the fragmentation and degradation of larger plastic items as a result of physical, chemical, and biological weathering processes (Auta et al., 2017). Environmental stressors such as ultraviolet radiation, mechanical abrasion, thermal stress, and oxidative reactions progressively weaken polymer structures, leading to the formation of microplastic particles over time (Ali et al., 2024). Urban and municipal sectors contribute through the degradation of consumer plastics, packaging materials, and construction products and releases during municipal solid waste collection (Horton et al., 2017). Transportation sectors generate secondary microplastics primarily via tyre and road-wear abrasion, which is now recognized as a major source of microplastic particles in terrestrial and freshwater environments (Kole et al., 2017; Wagner et al., 2018). Agricultural sectors contribute through the breakdown of plastic mulch films, greenhouse covers, irrigation pipes, and silage wraps, which act as long-term secondary sources of microplastics (Horton et al., 2017; Steinmetz et al., 2016). Fisheries and aquaculture sectors further contribute through the deterioration of plastic fishing gear, ropes, and nets, generating microplastic fragments that can be transported into freshwater systems (Ding et al., 2021). Collectively, these fragmented materials constitute the dominant source of microplastics detected in freshwater ecosystems (Li et al., 2018; Upadhyay et al., 2024).

Similarly, primary nanoplastics may also be intentionally produced for specialised applications, such as drug delivery systems, biomedical products, cosmetics, coatings, membranes and other advanced industrial uses. Secondary nanoplastics can be produced through the further breakdown of larger plastic debris and microplastics, particularly under prolonged weathering, abrasion, oxidation and microbial degradation (Jani et al., 2025).

Unless particle size is specifically discussed, the term “microplastics” is used in this introductory chapter as a broad term for small plastic particles commonly discussed in the freshwater pollution literature. Where relevant, nanoplastics are discussed explicitly.

1.3 Microplastics in freshwater systems: Inputs, entry pathways, transport, redistribution, accumulation, and transformation

1.3.1 Inputs and entry pathways of microplastics into freshwater ecosystems

Once generated, microplastics can be transferred into freshwater ecosystems through multiple entry pathways. Entry pathways describe the routes by which microplastics move from their sources into rivers, lakes, reservoirs, and wetlands (Fig. 1.1).

Urban waste streams containing microplastics are conveyed into freshwater systems primarily through urban drainage networks and sewage effluent (Ngo et al., 2019; Siegfried et al., 2017). In many developed countries, domestic wastewater and stormwater generated in urban areas are typically conveyed to centralized wastewater plants, where they undergo treatment before being released into downstream aquatic systems. However, synthetic textile fibres released during household laundering and microplastic beads used in cosmetic and personal care products may enter aquatic environments in regions lacking adequate sewerage or stormwater infrastructure or wastewater bypasses treatment processes, allowing microplastics to be discharged directly into receiving waters (Duis & Coors, 2016; Murphy et al., 2016). For the urban wastewater containing microplastics entering wastewater treatment plants (WWTPs), although WWTPs can remove a high proportion of microplastics, up to 95% overall, with tertiary treatment achieving removal efficiencies of 90% for particles larger than 10 μm, a large amount of microplastics is discharged into natural water through WWTPs (Miloloža et al., 2021; Talvitie et al., 2017). In addition, since a large amount of microplastics fraction is transferred into the activated sludge, the indirect pathway for microplastics released from WWTPs is WWTP sludge, which is used in green construction and as a fertilizer in fields (Sun et al., 2019). These microplastics could possibly enter freshwater environments during rainstorms or via agricultural or farming activities (Ding et al., 2021).

As for agricultural pathways, the use of plastic mulch films, greenhouse coverings, irrigation materials, and the application of sewage sludge contribute to microplastics entering freshwater ecosystems through surface runoff, subsurface drainage, and irrigation return flows (Singh et al., 2025; Zhao et al., 2022). In addition, microplastics can be transported into freshwater systems through rainfall and atmospheric deposition

(Aeschlimann et al., 2022; Dris et al., 2018).

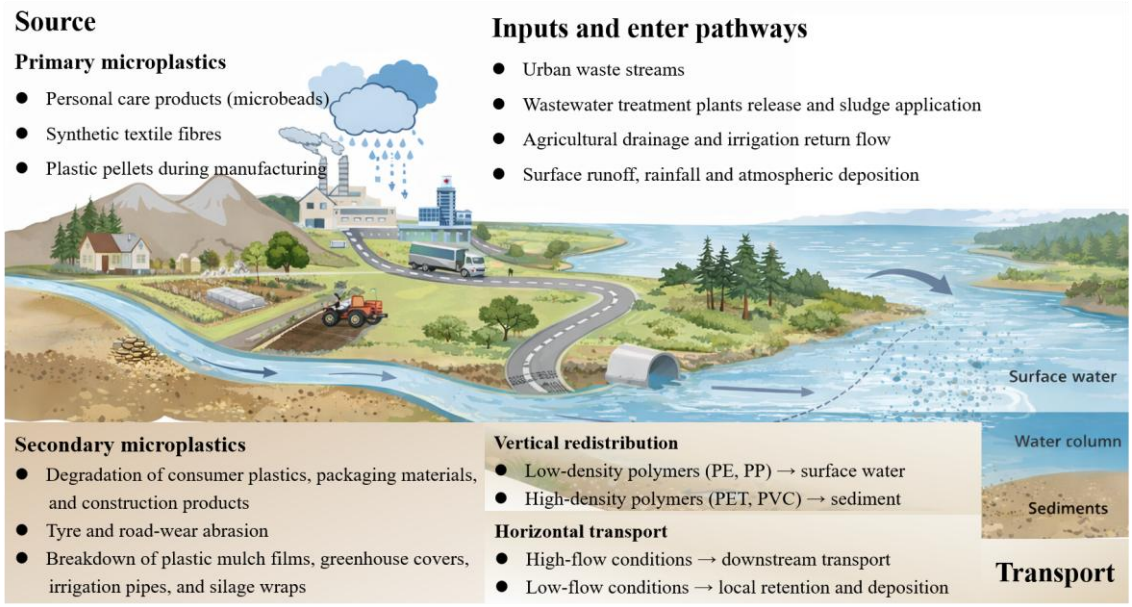


Figure 1.1. Sources, inputs, entry pathways and transport of microplastics in freshwater ecosystem.

1.3.2 Transport and redistribution of microplastics within freshwater ecosystems

Once released into freshwater systems, microplastics are subject to a series of physical, chemical, and biological processes that shape their distribution, transport pathways, and long-term fate (Atugoda et al., 2020; Cole et al., 2011). Transport occurs both horizontally, through river flow and hydrological connectivity, and vertically, through exchanges between surface waters, the water column, and sediments (Fig. 1.1). These processes determine the residence time of microplastics within freshwater systems and shape subsequent accumulation patterns.

Horizontal transport is primarily controlled by natural conditions, such as river discharge and flow velocity (Ding et al., 2021). Rivers act as major pathway, transferring microplastics from upstream catchments to downstream lakes, reservoirs, floodplains, and estuaries (Wagner et al., 2014). High-flow conditions promote downstream transport, whereas reduced flow velocities favour local retention and deposition (Adjornor et al., 2024; He et al., 2021). Episodic events such as storms and floods can further enhance transport by resuspending previously deposited microplastics and redistributing them

within the system (Hurley et al., 2018).

Vertical redistribution of microplastics is strongly influenced by microplastic properties (e.g., size, density, shape, colour, and polymer composition). Polymer density plays a key role in determining particle fate: low-density polymers such as polyethylene (PE) and polypropylene (PP) tend to remain at or near the water surface, whereas higher-density polymers, including polyethylene terephthalate (PET) and polyvinyl chloride (PVC), settle more readily into riverbeds and lake sediments (Ballent et al., 2013; Waldschläger & Schüttrumpf, 2020). Particle size and shape further influence settling behaviour and transport pathways. Smaller particles can remain in suspension for a longer time under comparable hydrodynamic conditions (Enders et al., 2015). Fibres typically exhibit lower settling velocities compared to compact fragments of similar size because of increased drag, enabling longer suspension times (Waldschläger & Schüttrumpf, 2019).

Environmental processes and biological interactions can modify microplastic properties during transport. Environmental degradation processes, including photodegradation, mechanical abrasion, and chemical weathering, further fragment larger plastic items, generating additional microplastic particles dispersed throughout freshwater systems (Liu et al., 2022a; Talbot & Chang, 2022). Biological interactions add additional complexity to microplastic fate. Microplastics can be entangled with phytoplankton, ingested by aquatic organisms, and colonized by microbial communities through biofilm formation, modifying the surface properties and density of microplastics, promoting aggregation and altering their environmental fate (Liu et al., 2022b; Raju et al., 2023; Shah et al., 2008). Thus, the dynamic interplay between microplastics and both abiotic and biotic components of freshwater systems governs their environmental behaviour and contributes to their persistence across diverse aquatic compartments.

1.3.3 Accumulation and abundance of microplastics in freshwater ecosystems

Freshwater ecosystems exhibit substantial spatial and temporal variability in microplastic abundance, reflecting the combined influences of source intensity, hydrological transport, particle characteristics, and environmental retention processes (Horton et al., 2017; Wagner et al., 2014). Consistent with the transport mechanisms

outlined in Section 1.3.2, rivers, lakes, and reservoirs function not only as conduits for microplastic movement but also as zones of transient or long-term accumulation, depending on local physicochemical conditions (Koelmans et al., 2019).

1.3.3.1 Surface water

Microplastics have been detected across freshwater environments on all continents, yet their abundance varies widely among regions (Table 1.1) (Wang et al., 2021b). To reflect recent advances in sampling and analytical methodologies, the studies summarised in Table 1.1 focus primarily on work published since 2019, while earlier findings have been comprehensively synthesised in existing review articles (Horton et al., 2017; Koelmans et al., 2019; Wagner et al., 2014). Most studies have reported microplastic abundance using number-based units (i.e., particles per unit volume or mass), whereas fewer studies used weight-based units (e.g., grams per litre) (Li et al., 2020a). To date, the highest reported microplastic concentration in surface water was 3622000 particles/m^3 (Kinnickinnic River, United States) or 2.5803 g/L (Maribyrnong and Yarra Rivers, Australia) (Fam et al., 2017; Simmerman & Coleman Wasik, 2019). Other freshwater systems where there have been reports of severe microplastic pollution include Taihu Lake (China), the Saigon River (Vietnam), Winnipeg Lake (Canada), and Maribyrnong and Yarra Rivers (Australia).

This pronounced heterogeneity is closely linked to hydrodynamic conditions and catchment characteristics. High flow velocities and turbulent conditions promote downstream transport and dilution, whereas low-energy environments favour local retention and accumulation (Adjornor et al., 2024; Talbot & Chang, 2022). Rainfall events can potentially increase surface-water microplastic concentrations by mobilizing particles from urban surfaces and riverbanks, consistent with storm-driven wash-off mechanisms discussed in Section 1.3.1 (Piñon-Colin et al., 2020; Xia et al., 2020). Proximity to urban centres, population density, wastewater discharge, and land-use intensity are repeatedly identified as strong predictors of elevated microplastic abundance in surface waters (Kunz et al., 2023; Talbot & Chang, 2022). In addition, it is critical to acknowledge that the concentration of microplastics in each study differs in terms of sampling methods.

Table 1.1. Example concentrations, polymer types and sizes of microplastics reported in freshwater environments worldwide since 2019. The table is illustrative rather than exhaustive and highlights the variability of reported microplastic abundances across freshwater ecosystems.

Area	Site	Concentration	Type	Size	References
China	Pearl River	Urban section: 19860 items/m^3	PA, cellophane, PP, PE	< 0.5 mm	(Yan et al., 2019)
	Lower Yellow River near the estuary	Dry season: 930000 items/m^3; Wet season: 497000 items/m^3	PE, PP, PS	< 5 mm	(Han et al., 2020)
	Yangtze River Basin	1270 items/m^3	PP, PE, PA, PS, PVC, PET, PC, PVF	<1.0 mm	(Yuan et al., 2022)
	Three Gorges Reservoir	4703 ± 2816 items/m^3	PS, PP, PE	< 5 mm	(Di & Wang, 2018)
	Shahe River	1772 to 3877 items/m^3	PET, PP	0.01 - 0.3 mm	(Zhang et al., 2022a)
	Taihu Lake	3400 to 25800 items/m^3	CP, PET, PP, polyester	100 - 1000 μm	(Su et al., 2016)
	Maozhou River	4000 to 25500 items/m^3	PE, PP, PS, PVC	0.1-1 mm	(Wu et al., 2020)
	Poyang Lake	5000 to 34000 items/m^3; Average concentration: 0.2034 g/L	PP, PE, PA, PVC	< 0.5 mm	(Yuan et al., 2019)
	Wei river	3670 to 16700 items/m^3; Average concentration: 0.918 g/L	PE, PVC, PS	< 5 mm	(Ding et al., 2019)
Vietnam	Saigon River	172000 to 519223 items/m^3	PE, PP, PET	< 5 mm	(Lahens et al., 2018)
Indonesia	Cisadane River	44670 items/m^3	PE, PS, PP	< 5 mm	(Sulistyowati et al., 2022)
	Surakarta city river basin	25500 to 52200 items/m^3	PS, silicone polymer, polyester, PA	0.3 - 5 mm	(Ismanto et al., 2023)
Russia	Degtyarka Lake	26000 items/m^3	PVC	10 - 960 nm	(Malygina et al., 2021)
Canada	Winnipeg Lake	1933000 items/m^3; Average concentration: 1.7397 g/L	N/A	< 5 mm	(Anderson et al., 2017)

Area	Site	Concentration	Type	Size	References
United States	Kinnickinnic River	545,000 to 3,622,000 items/m^3	N/A	< 0.05 mm	(Simmerman & Coleman Wasik, 2020)
	Gallatin River	1200 items/m^3	Semi-synthetic cellulose, polyester	< 5 mm	(Barrows et al., 2018)
	Hillsborough River	440 to 9700 items/m^3; Average concentration: 2.36 mg/m^3	PE, PP, PS	< 0.5 mm	(Baldwin et al., 2020)
Australia	Maribyrnong and Yarra Rivers	2867000 items/m^3; Average concentration: 2.5803 g/L	N/A	< 2 mm	(Fam et al., 2017)
United Kingdom	Thames River	24.8 items/m^3	N/A	< 5 mm	(Rowley et al., 2020)
Switzerland	Rhine River	0.04–9.97 items/m^3	PE, PP, PS	< 5 mm	(Mani & Burkhardt-Holm, 2020)
Germany	Tollense River	1357 to 2146 items/m^3	N/A	< 5 mm	(Tamminga et al., 2022)
Netherlands	Amsterdam canals	100333.3333 items/m^3	N/A	< 5 mm	(Leslie et al., 2017)
Italy	Garda Lake	25000 items/m^3	PP, PE, PS	< 5 mm	(Sighicelli et al., 2018)
South Africa	Orange-Vaal River	230 ± 270 items/m^3	N/A	< 5 mm	(Weideman et al., 2019)
Antarctica	Ballenas Stream	0.95 items/1000 m^3	Polyester, acrylic, PTFE	< 5 mm	(González-Pleiter et al., 2020)

Note: PA, polyamide; PS, polystyrene; PTFE, polytetrafluoroethylene; PVF, polyvinyl fluoride; CP, cellophane

1.3.3.2 Freshwater sediments

In contrast to surface waters, freshwater sediments act primarily as long-term sinks for microplastics, playing a central role in regulating their environmental storage and remobilization (Wang & Jönsson, 2018; Zhang et al., 2020). Globally, reported microplastic abundances in freshwater sediments have ranged from dozens to tens of thousands of particles per kilogram (Table 1.2). The highest recorded microplastic concentration in sediment to date is 335714 items/kg from Huron Lake (United States). In China, the average concentration has ranged from 20 to 32,947 particles/kg. Sediment accumulation patterns are strongly governed by particle-specific properties and hydrodynamic sorting processes described in Section 1.3.2. High-density polymers (e.g., PET, PVC) and smaller particle sizes are preferentially deposited in sediments, while biofouling and aggregation processes promote the downward transport of initially buoyant polymers (Lenaker et al., 2019; Rummel et al., 2017). Although freshwater sediments act as long-term sinks, they do not represent permanent endpoints. Resuspension of sediment-bound microplastics can occur during high-flow events, flooding, dredging, or bioturbation, allowing particles to re-enter the water column and sustain prolonged exposure within freshwater ecosystems (Jiang et al., 2025).

Table 1.2. Example concentrations, types and sizes of microplastics found in different areas of freshwater sediments since 2019. The table is illustrative rather than exhaustive and highlights the variability of reported microplastic abundances across freshwater sediments.

Area	Site	Concentration	Type	Size	References
China	Rivers in the Tibetan Plateau	90 to 130 items/kg	PET, PA, PE, PS	>70% <1 mm	(Jiang et al., 2019a)
	Wen-Rui Tang River	20 to 5000 μm：32947 ± 15342 items/kg 300 to 5000 μm：4635 ± 2107 items/kg	PE, PP, PS, PES	< 5 mm	(Zhang et al., 2020)
	Wei River	360 to 1320 items/kg	PE, PVC, PS	< 0.5 mm	(Ding et al., 2019)
	Danjiangkou Reservior	708 to 3237 items/kg	PA, PE, PP	< 5 mm	(Lin et al., 2021)
	Poyang Lake	54 to 506 items/kg	PP, PE	57.1% < 0.5 μm	(Yuan et al., 2019)
India	Brahmaputra River	531 to 3485 items/kg	PP, PE, PA, PET, PVC, PTFE	150 μm - 5 mm	(Tsering et al., 2021)
Pakistan	Rawal Lake	104 items/kg	PE, PP, polyester, PET, PVC	< 5 mm	(Irfan et al., 2020)
Russia	Onego-Shuya River inflow Lake	2188.7 items/kg	N/A	< 5 mm	(Zobkov et al., 2020)
United States	Huron Lake	59 to 335714 items/kg	Anthropogenic, proteinaceous, PET, acrylonitrile, PP, nylon	< 5 mm	(Belontz et al., 2022)
	Erie Lake	431 items/kg	Cellulose, PP, PVC	< 5 mm	(Lenaker et al., 2021)
Canada	Thames River	60 to 24440 items/kg	Cellulose, PE, PP, PET	0.18-8.70 mm	(Corcoran et al., 2020)
Australia	Brisbane River	10 to 520 items/kg	PE, PA, PP	< 3 mm	(He et al., 2020)
Germany	Tollense Lake	10476 items/kg	PE, PA, PP	< 0.5 mm	(Hengstmann et al., 2021)

Area	Site	Concentration	Type	Size	References
United Kingdom	Kelvin River	296500 items/m^3; Average concentration: 0.26685 g/L	N/A	0.011 - 2.8 mm	(Blair et al., 2019)
Hungary	Tisza River	3177 items/kg	N/A	< 0.5 mm	(Kiss et al., 2021)
South Africa	Braamfontein River	166.8 items/kg	N/A	< 5 mm	(Dahms et al., 2020)

Note: PU, polyurethane.

The accumulation of microplastics in freshwater sediments therefore raises particular concern due to their persistence, selective enrichment of certain polymer types, and potential for long-term remobilisation. By functioning as both reservoirs and secondary sources, sediments play a critical role in regulating microplastic exposure for benthic organisms and in influencing the long-term fate of microplastics within freshwater ecosystems. These characteristics underscore the importance of sediments as an important component in freshwater microplastic risk assessment, which is explored further in Section 1.3.

1.3.4 Transformation and weathering of microplastics in freshwater ecosystems

Microplastics present in freshwater ecosystems are subject to continuous transformation through physical, chemical, and biological weathering processes, which modify particle size, surface properties, and density, thereby influencing transport behaviour, accumulation patterns, and environmental persistence (Li et al., 2022a; Luo et al., 2025).

Physical transformation occurs through interactions with the surrounding environmental forces, such abrasion against gravels and other surfaces, leading to progressive fragmentation of microplastics into smaller and more fragile particles (Enfrin et al., 2019; White & Turnbull, 1994). Chemical transformation is driven primarily by photoinduced reactive oxygen species (ROS), which weaken polymer structures and facilitate further fragmentation (He et al., 2023). Biological processes also contribute to microplastic transformation. Microplastics are rapidly colonized by microorganisms, forming biofilms that alter surface chemistry and increase effective particle density (Liu & Zheng, 2025). Importantly, these processes of transformation can alter microplastic behaviours within freshwater systems (Duan et al., 2021). Understanding microplastic transformation is therefore essential for interpreting observed distribution patterns and for assessing their ecological relevance.

1.4 Biological responses of micro- and nanoplastics in freshwater ecosystem

Although much of the early experimental evidence on microplastic toxicity has been derived from marine organisms, these studies have provided critical mechanistic insights that are broadly applicable across aquatic ecosystems. Study by Galloway et al. (2017) demonstrated that microplastics ingested by aquatic species induced a serious of adverse outcomes, including growth inhibition, reproductive decline, and reduced offspring viability. Microplastics can affect freshwater ecosystems at multiple levels, from physicochemical properties of water and sediments to organisms, communities, and ecosystem processes. Their impacts include direct toxicity to biota, alteration of habitat conditions, disruption of biogeochemical cycles, and potential impairment of ecosystem structure and function (Gao et al., 2025; Kye et al., 2023; Morales-Espinoza et al., 2025; Raju et al., 2023; Wang et al., 2021a; Wong et al., 2020). The following subsections summarize current knowledge on the effects of microplastics on freshwater benthic environments, key organism groups, and ecosystem-level properties.

1.4.1 Effects of micro- and nanoplastics on the benthic environments

In freshwater ecosystems, the benthic environment comprises the sediment matrix together with associated microorganisms and benthic organisms that interact at the sediment-water interface. Microplastics are transported from the overlying water column to sediments through a combination of hydrodynamic sorting, biofouling-induced changes in buoyancy, particle-specific properties (e.g., density, size, and shape), and biological processes such as ingestion, faecal pellet production, and bioturbation (Kooi et al., 2017). Once deposited, microplastics can interact simultaneously with sediments, pore water, microorganisms, plants, and benthic fauna. As such, freshwater sediments function not only as transient and long-term sinks but as dynamic interfaces that regulate microplastic storage, remobilization, and bioavailability within benthic environments (Yang et al., 2021).

Microplastics accumulated in sediments have been shown to alter the chemical and biophysical characteristics of the sediments, with cascading effects on sediment ecosystem functions (Fazey & Ryan, 2016). First, microplastic inputs can disrupt

sediment carbon storage and biogeochemical conversion processes. Most microplastics are composed primarily of carbon-based polymers, and their incorporation into sediment introduces exogenous carbon pools that interact with natural sediment carbon cycling (Jiang et al., 2019b). Several studies have demonstrated that microplastic addition leads to changes in total carbon and total organic carbon and can influence greenhouse-gas-related processes, with such effects mediated by shifts in microbial communities and functional genes (Li et al., 2022b; Liu et al., 2017; Zhang et al., 2024b). Second, microplastics induce significant alterations to sediment physicochemical properties, such as pH, conductivity and nutrient (e.g., nitrogen) content (Li et al., 2024a; Zhang et al., 2015). These physicochemical changes in turn act as a cascade trigger for shifts in sediment enzymatic activities and microbial community composition (de Souza Machado et al., 2018; Fei et al., 2020). Sediment microbial activity plays an important role in carbon and nitrogen fixation, and the decomposition of organic matter; meanwhile, microbiota around plant roots can regulate sediment enzyme activity and influence plant growth (Qiu et al., 2022b). Microplastics disrupt these processes by modifying microbial abundance, specifically reducing the diversity and richness of sediment microbial communities and destabilizing bacterial community structure (Li et al., 2022b; Li et al., 2024a; Qiu et al., 2022b). Thus, the impacts of microplastics on sediment environmental characteristics and microbial diversity can disrupt the carbon and nitrogen cycle and hinder plant growth, ultimately undermining the ecosystem functions of sediments.

At the organismal level, benthic organisms play an important role in sustaining freshwater ecosystem balance and nutrient cycling by connecting primary producers to higher trophic levels (Zhang et al., 2023). Micro- and nanoplastics can cause various effects on benthic organisms, encompassing physical damage, oxidative stress, impaired reproductive function and disrupted energy metabolism (Gong et al., 2023; Liang et al., 2023). For the impacts on survival and growth, studies have shown that PP microplastics increased mortality of *Hydropsyche pellucidula*; the survival rate of *Macrobrachium nipponense* decreased with increasing PS microplastic concentration and exposure time (Li et al., 2021; Piccardo et al., 2021). A significant growth reduction was observed on veliger larvae of Slipper Limpets (*Crepidula onyx*) when exposed to PS microplastics (Lo

& Chan, 2018). In addition, microplastic exposure can cause pathological changes, including gut damage, gut cilia deformation, and degeneration of digestive tubules (Fu et al., 2022; Li et al., 2020b). Several studies demonstrated that PS microplastics increased superoxide dismutase (SOD) and catalase (CAT) activities in freshwater molluscs, indicating the production of oxidative stress (Roy et al., 2023; Wang et al., 2020c). PS microplastic exposure decreased acetylcholinesterase (AChE) activity rapidly and significantly in bivalve (*Mytilus galloprovincialis*) gill tissues, suggesting their neurotoxicity on benthic organisms. Chronic PE microplastics exposure resulted in a significant decrease in reproduction of *Hyalella azteca* (Au et al., 2015).

Despite evidence showing that microplastics affect individual components of benthic environments, including sediment properties, microbial communities, and benthic organisms, existing studies largely examine these effects in isolation or under simplified experimental conditions. Therefore, the extent to which microplastic-induced changes in sediments and microorganisms interact with other organisms and influence ecosystem processes remains insufficiently understood.

1.4.2 Biological responses and potential toxicity mechanisms of micro- and nanoplastic on freshwater organisms

1.4.2.1 Conceptual distinction between organismal stress and organismal toxicity

In this thesis, organismal stress is defined as a physiological, biochemical, microbial or metabolic response that indicates disturbance of homeostasis and may be adaptive or reversible. Stress-related responses include altered reactive oxygen species (ROS), antioxidant enzyme activity, lipid peroxidation markers, immune-related indicators, gut microbiota composition, metabolomic profiles and enzyme activity changes. These endpoints are useful early-warning indicators of biological disturbance, but they do not necessarily demonstrate irreversible toxicity (Barton et al., 2002).

Organismal toxicity is defined as a direct, measurable and potentially irreversible adverse effect on organismal performance, tissue integrity, survival or fitness. Toxicity-related adverse outcomes include mortality, sustained growth inhibition, severe histopathological damage, impaired feeding, persistent reproductive impairment and

reduced offspring viability. Some endpoints may fall between these two categories. For example, reduced egg production, apoptosis-related enzyme activity or reduced photosynthetic pigment content may indicate potential adverse outcomes, but their interpretation depends on whether they are supported by evidence of persistence, functional impairment, recovery failure or longer-term fitness consequences (Ankley et al., 2010).

This distinction is important for interpreting micro- and nanoplastic studies because many studies rely on biomarkers, microbiome analysis and metabolomics. These endpoints are sensitive to biological disturbance, but they are not equivalent to direct toxicity unless they are linked to adverse organismal outcomes. Therefore, in this thesis, biomarker, microbiome and metabolomic changes are interpreted primarily as stress-related responses, whereas endpoints related to survival, growth, tissue integrity, feeding, reproduction and offspring viability are interpreted as toxicity-related adverse outcomes where the evidence supports this conclusion.

1.4.2.2 Effects of microplastics on fish

Freshwater fish are ecologically, economically, and nutritionally important components of freshwater ecosystems. They are often used as significant indicators of microplastic pollution and are valuable for studying their ecological and behavioural impacts across different biological scales (Ghosh et al., 2025). Fish can encounter microplastics through contact with gills or skin; however, oral ingestion is generally considered the primary route of entry into the body (Abbasi et al., 2018; Kurniawan et al., 2023; Park et al., 2020). Ingested microplastics are retained mainly in the gastrointestinal tract, where they may be partially fragmented, interact with gut contents, and eventually be excreted (Jabeen et al., 2017). Microplastics excreted in faecal matter may act as secondary sources of microplastics for benthic or pelagic organisms, thereby contributing to trophic transfer within freshwater food webs (Hoang & Felix-Kim, 2020). Smaller particles, including small microplastics and nanoplastics, may have the potential to cross the gut barrier, enter the bloodstream, and accumulate in other organs, including the liver, kidneys, gut and even the brain of fish, although translocation depends on chemical

properties, sizes, and shapes (Ribeiro et al., 2019).

Microplastics may induce physical damage through mechanical physical interactions. Physical damage mainly involves internal abrasions and obstructions in the digestive system. Microplastics induce abrasion, lesions, and inflammation of the digestive tract, disrupting normal digestive function and impairing nutrient absorption (Banaee et al., 2025). For example, ingestion of PE has been shown to significantly reduce food intake in the goby fish *Pomatoschistus microps*, a response attributed to digestive organ blockage by the particles (de Sá et al., 2015). In zebrafish, PS microplastics have been shown to cause gut villi rupture and mucosal damage (Lu et al., 2016). Exposure to microplastics (PA, PE, PP, and PVC) induces gut damage in zebrafish, including villus dehiscence and enterocyte lysis (Lei et al., 2018). Once internalized, microplastics can cause gastrointestinal blockage, tissue inflammation, and organ damage, as well as impair nutrient absorption and energy balance, posing significant health risks to fish (Alak et al., 2022; Wang et al., 2024).

Exposure to microplastics may lead to either stress-related responses or direct toxicity-related adverse outcomes, depending on their severity, persistence and biological consequence. Stress-related responses include physiological, biochemical, microbial or metabolic disturbances, such as oxidative stress, gut microbiota dysbiosis, metabolic disorders, and immune response, which indicate disturbance of normal biological function; while direct toxicity-related adverse outcomes include effects that more clearly impair organismal performance, tissue integrity or fitness, such as inhibition of growth and reproduction, severe tissue damage, impaired nutrient absorption, organ damage or mortality (Jiao et al., 2025; Jovanović, 2017; Naidoo & Glassom, 2019). Notably, the liver emerges as a primary target organ for microplastic-induced effects. Microplastics trigger hepatic inflammatory responses and histological damage, characterized by vacuolation, nuclear pyknosis or peripheral nuclei (Sun et al., 2024). Beyond hepatocellular injury, microplastic exposure induces oxidative stress and alters metabolic reactions in the liver (Lu et al., 2016). The known stress-related responses and toxicity-related adverse outcomes of micro- and nanoplastics on fish are summarized in Table 1.3.

Table 1.3. The known stress-related responses and toxicity-related adverse outcomes of micro- and nanoplastics on fish. The table is illustrative rather than exhaustive and lists the reported stress-related responses and toxicity-related adverse outcomes of different types of micro- and nanoplastics on fish**.**

Type	Species	Concentration, size and exposure time	Reported biological responses	References
PE	*Oreochromis niloticus*	10 mg/L; 15d	Reduced red blood cells, haemoglobin, neutrophils, lymphocytes, and antioxidant capacity	(Sabra et al., 2024)
PE	*Gambusia holbrooki*	100, 200 µg/L; 200-250 µm; 14d	Increased antioxidant enzyme levels	(Banaee et al., 2023)
PE	*Cyprinus carpio*	100 mg/L; 100 nm to 5 mm; 15 d	Decreased haematological parameters level; oxidative stress; immune destruction	(Hamed et al., 2022)
PS	*Anabas testudineus*	13.6, 23.6 mg/L; 14 µm; 15d	Oxidative stress; histological damage; altered metabolic functions in the mitochondrial fractions of kidney and muscle tissues	(Arya et al., 2025)
PS	*Cyprinus carpio*	1 µg/L; 8 µm; 21d	Inflammatory injury in hepatopancreas; endoplasmic reticulum stress; autophagy and apoptosis.	(Cui et al., 2024)
PS	*Channa argus*	500, 1000, 2000 ng/L; 8 µm; 28d	Inflammation, apoptosis, and autophagy in the liver, kidney, intestine and gill; oxidative stress	(Jiao et al., 2025)
PP	*Cyprinus carpio*	1.0, 2.5 g/L; 0.9 mm; 7d	Oxidative damage, increased malondialdehyde and glutathione levels	(Yedier et al., 2023)
PVC	*Oreochromis niloticus*	10 mg/L; < 100 nm; 4d	Oxidative stress; increased levels of plasma total proteins, albumin, globulin, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, creatinine, and uric acid	(Soliman et al., 2025)
PE, PS	*Oryzias latipes*	4, 4000 µg/L; 2, 20,200 µm; 24d	Altered immune response; changes in metabolic function	(Liu et al., 2021)
PE, PVC, PS	*Ambassis dussumieri*	1.769 µg/L; 250 to 1000 µm; 92d	Decreased growth, survival and body condition	(Naidoo & Glassom, 2019)

As a representative vertebrate in aquatic ecosystems, zebrafish have emerged as the most widely used model organism for investigating the effects of micro- and nanoplastics in freshwater systems due to their small size, low breeding costs, short reproductive cycles, rapid generation turnover, and external fertilization (Li et al., 2023a). Zebrafish are particularly suitable for assessing biological responses across multiple levels of organisation, including particle uptake, tissue distribution, oxidative stress, immune-related responses, gut microbiota alteration, metabolic disturbance, behaviour, development and reproduction. Fig. 1.2 illustrates the reported stress-related and toxicity-related endpoints of micro- and nanoplastics in zebrafish, including oxidative stress, immunotoxicity-related responses, gut damage, metabolome alteration, neurotoxicity and locomotor toxicity, genotoxicity, reproductive toxicity and developmental toxicity (Bhagat et al., 2020). Micro- and nanoplastics can accumulate in multiple tissues, such as the gut, liver, gills, muscle, gonads, and brain (Lu et al., 2016; Pitt et al., 2018a; Veneman et al., 2017).

Previous research has demonstrated that PS microplastics can bioaccumulate in adult zebrafish and be maternally or paternally transferred to offspring, inducing intergenerational adverse effects (Pitt et al., 2018b). The biological effects of microplastics may differ among life stages. In zebrafish embryos, nanoplastics have been reported to increase mortality and induce developmental malformations, such as pericardial and yolk sac oedema (Sökmen et al., 2020); in adult zebrafish, gut-accumulated microplastics may trigger false satiety signals and direct gut damage. Furthermore, research has shown that microplastics can induce stress-related microbial and metabolic disturbance, including gut microbiota dysbiosis and metabolic homeostasis, further compromising gut health (Qiao et al., 2019b). In the liver, microplastics cause hepatocellular injury, metabolic dysfunction, and ROS mediated oxidative stress (Lu et al., 2016; Umamaheswari et al., 2021). Among these endpoints, hepatocellular injury and severe histological alteration provide stronger evidence of toxicity-related adverse outcomes, whereas oxidative stress and metabolic changes mainly indicate stress-related physiological disturbance.

A growing body of research highlights the gut-liver axis, a bidirectional regulatory

network integrating signals from the gut and liver, modulated by dietary, genetic, and environmental factors, as a critical target in micro- and nanoplastic-related biological responses (Tilg et al., 2022; Zhang et al., 2024a). For example, Feng et al. (2021) reported that PS microplastics induced adverse effects in the marine medaka (*Oryzias melastigma*) primarily via disrupting the gut-liver axis and perturbing liver-gut microbiota co-metabolism. Similarly, Zheng and Wang (2025) found that PS microplastic exposure triggered hepatic metabolic disturbances in Nile tilapia (*Oreochromis niloticus*), with these effects tightly regulated by gut microbiota dysbiosis. These studies suggest that gut microbiota alteration and hepatic metabolic disturbance may be important stress-related mechanisms linking particle exposure to broader physiological effects.

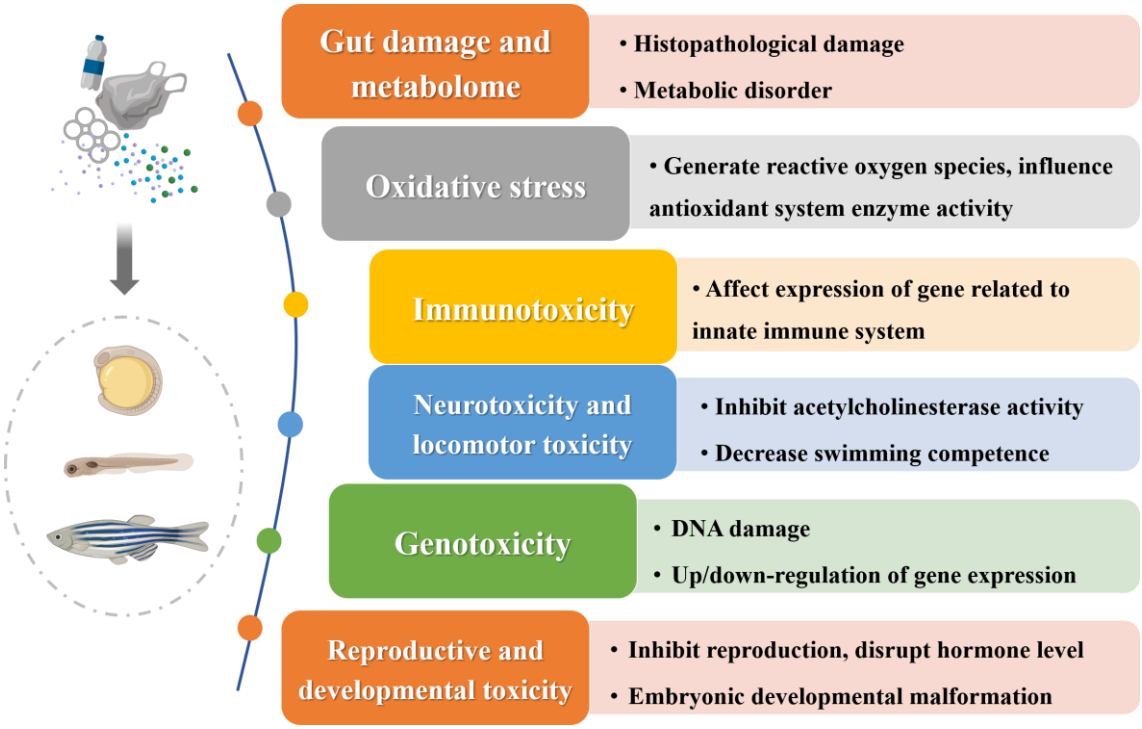


Figure 1.2. Reported stress-related responses and toxicity-related adverse outcomes of micro- and nanoplastics in zebrafish, including gut damage, metabolome alteration, oxidative stress, immunotoxicity-related responses, neurotoxicity and locomotor toxicity, genotoxicity, reproductive toxicity and developmental toxicity.

Despite the growing evidence that microplastics interfere with antioxidant defences, gene expression, hormone secretion, metabolic pathways, and gut microbiota in

freshwater fish, gender-specific effects remain insufficiently explored. This oversight is notable given that gender-specific effects of microplastics have been documented in other organisms. For example, microplastics have been shown to induce gender-specific sensitivity in antioxidant and detoxification systems in *Daphnia magna*, while PS microplastics disrupt the hypothalamic-pituitary-gonadal axis and steroidogenic pathways of the marine medaka (*Oryzias melastigma*) in a gender-dependent manner, disturbing sex hormone balance, delaying gonadal maturation, and impairing early offspring development (Lee et al., 2021; Wang et al., 2019). Such gender-differentiated responses to microplastic exposure can lead to unequal health impacts between males and females and ultimately influence reproductive success, offspring viability, and population stability (Wang et al., 2019). Therefore, investigating the gender-specific effects of microplastics on freshwater fish is essential for elucidating how these pollutants influence not only individual health but also population dynamics and the long-term stability of freshwater ecosystems.

1.4.2.3 Effects of microplastics on zooplankton, algae and plants

Zooplankton represent key primary consumers in freshwater food webs and constitute an important trophic link between primary producers and higher trophic levels (Swain et al., 2025). They can ingest microplastics directly or through consumption of microplastic-contaminated phytoplankton (Swain et al., 2025). Microplastic ingestion can lead to various detrimental effects in zooplankton, including reduced feeding efficiency and energy supply, induced oxidative stress, altered growth rates, and increased mortality (Chenxi et al., 2022; Cole et al., 2019; Jeyavani et al., 2022; Liu et al., 2020; Yu & Chan, 2020). Notably, reduced feeding efficiency and energy supply can further cascade to affect zooplankton reproduction and survival (Van Colen et al., 2020).

Algae and plants are primary producers in the freshwater ecosystem and play an important role in the maintenance of habitats, and all their changes affect the structure and function of ecosystems (Allan et al., 2021; Chang et al., 2014). As shown in Fig. 1.3, microplastics exert adverse effects on algae, including growth inhibition, reduced photosynthetic efficiency, and induction of oxidative stress (Li et al., 2025a).

Many studies have identified the key mechanisms underlying these impacts: first, microplastics adsorb to the surface of algal cells, and this surface association reduces photosynthesis through two primary pathways: (1) a shading effect that limits light penetration to photosynthetic apparatus, and (2) physical obstruction of cell surfaces, which hinders material exchange and decreases CO_2 assimilation (Mao et al., 2018; Qiu et al., 2022a). These disruptions ultimately impair essential algal metabolic processes and constrain growth. Second, microplastics alter algal photosynthesis by targeting photosynthetic pigments (e.g., chlorophyll a, carotenoids) and regulating the expression of photosynthesis-related genes, for example, reducing the transcription of genes encoding light-harvesting complex proteins or Rubisco, a key enzyme in carbon fixation (Cunha et al., 2020; Karimi et al., 2024; Zhang et al., 2022c).

Beyond surface-associated effects, direct contact between micro- and nanoplastics and algal cells causes physical damage (e.g., cell wall abrasion, membrane rupture), compromising cellular structural integrity and triggering oxidative stress (Nigam et al., 2022). Apart from direct physical disturbance, microplastics can disrupt algal physiology by interfering with nutrient absorption, photosynthesis, and cellular respiration, indirectly elevating ROS production, leading to oxidative damage to lipids, proteins, and nucleic acids (Karalija et al., 2022). Notably, for algae involved in harmful cyanobacterial blooms, microplastics can modulate the production and release of algal toxins, posing heightened risks to freshwater ecosystem health and drinking water safety (Huang et al., 2024; Liu et al., 2025). Earlier work has shown that PS microplastics stimulated production and release of microcystins by *Microcystis aeruginosa* (Wu et al., 2023), consistent with the findings of Zheng et al. (2023).

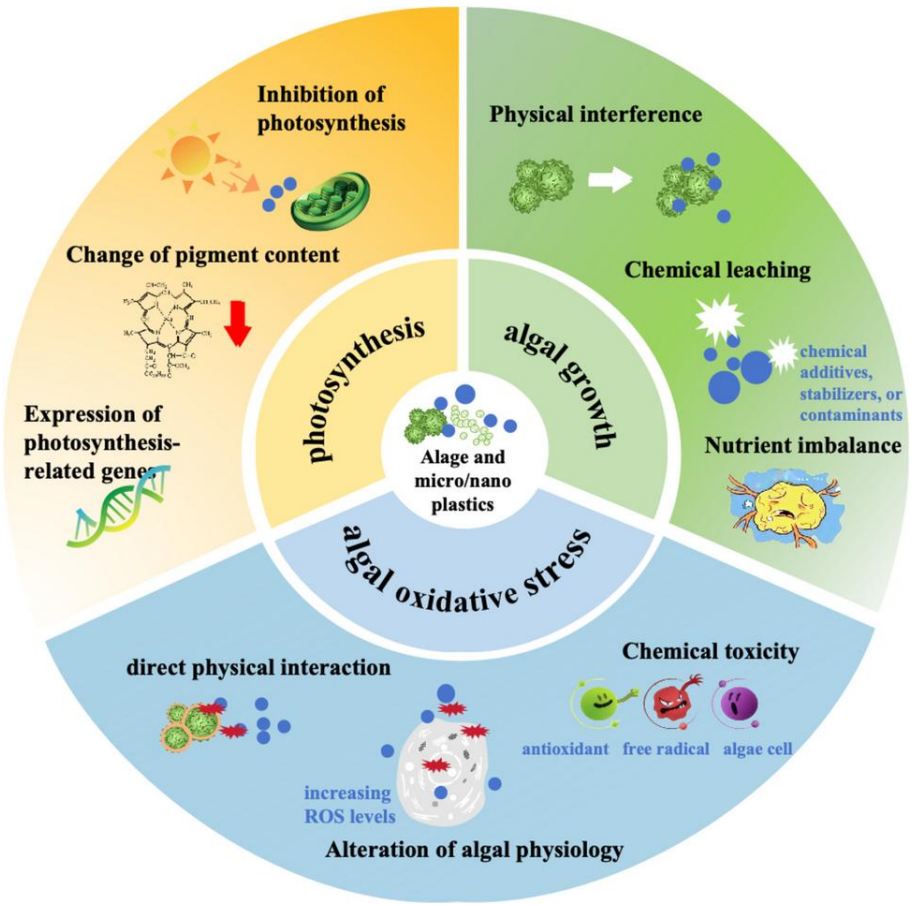


Figure 1.3. The known effects of microplastics on algal growth, photosynthesis, and oxidative stress parameters (Reprinted from (Li et al., 2025a)).

Some studies have reported contrasting results - microplastics can promote algal growth and photosynthesis or exert no observable effects (Table 1.4). Li et al. (2023b) found that PS nanoplastics enhanced the growth and physiological features of microalga *Scenedesmus quadricauda*. Another study reported that PE microplastics can promote growth of *Raphidocelis subcapitata* by acting as substrates of algal growth (Canniff & Hoang, 2018). In addition, Seoane et al. (2019) found that microplastics have no significant impact on algal growth due to the oil body produced by algae.

For freshwater plants, microplastics exhibit similar adverse effects to those observed in algae: microplastics readily adhere to plant surfaces, particularly in rooted, sediment-dwelling species or surface-attached plants, thereby impeding effective photosynthesis, growth, and reproduction (Chen et al., 2024). Direct micro- and nanoplastic exposure induces significant perturbations to plant physiological traits, including reduced root length, diminished root cell viability, decreased biomass accumulation, and suppressed

growth rates (Kalčíková et al., 2017; Li et al., 2024b; van Weert et al., 2019; Zhang et al., 2022b). For floating and submerged plant species, microplastics that sorb to leaf surfaces or become internalized further disrupt photosynthetic function by altering photosynthetic pigment content and inhibiting light absorption and carbon fixation (Chen et al., 2025; Li et al., 2024b; Rozman & Kalčíková, 2022).

Another critical stress-related impact is nanoplastic-induced oxidative stress (Ma et al., 2022). Excessive ROS accumulate in cells, triggering lipid peroxidation and further exacerbating photosynthetic inhibition, ultimately leading to irreversible damage to cell membranes and impaired cellular function (Lian et al., 2021). Beyond direct physiological harm, microplastics can affect the survival and function of plants indirectly by altering nutrient uptake and availability in freshwater ecosystem (Zeb et al., 2024). Microplastics can affect the uptake of nutrients by altering water properties (such as pH) and adsorbing nutrients (such as carbon, nitrogen, and phosphorus) (Wang et al., 2020a; Wu et al., 2019).

Table 1.4. The known effects of micro- and nanoplastics on algae and plants. The table is illustrative rather than exhaustive and lists the reported toxicity of different types of micro- and nanoplastics on algae and plants.

Type	Species	Concentration and exposure time	Size	Reported biological responses	References
PS	*Spirulina platensis*	0, 0.1, 1, and 10 mg/L; 20d	100 to 300 nm	Decrease of growth rate, dry weight, chlorophyll a and carotenoid levels; oxidative stress	(Karimi et al., 2024)
PS	*Navicula* sp.	0, 1, 3, 5, 8, and 10 mg/L; 48h	50 nm	Growth inhibition; reduced photosynthetic energy, light absorption, and light-harvesting capabilities; increase of antioxidant enzyme activity; changes in gene expression	(Li et al., 2025c)
PS	*Microcystis aeruginosa*	0, 0.1 and 0.5 mg/mL; 131d	6 μm	Inhibition of growth and phycobiliprotein content; increase of extracellular organic matter and microcystin content	(Liu et al., 2025)
PS	*Scenedesmus quadricauda*	0, 10, 25, 50, 100, and 200 mg/L; 14d	100 nm	Growth promotion; Increase of chlorophyll, soluble protein, and polysaccharide content and antioxidant enzyme activities	(Li et al., 2023b)
PS	*Vallisneria natans*	0, 10, 20, 50 mg/L; 24d	<10 μm	Increase of chlorophyll a and b content; oxidative stress; organelle damage of leaf cells; changes in microbial community on the leaf biofilms	(Zhang et al., 2022b)
PS	*Utricularia vulgaris*	0, 15, 70 and 140 mg/L; 7d	1, 2 and 5 μm	Inhibition of relative growth rates and the functional traits of leaves; oxidative damage	(Yu et al., 2020)
PS	*Vallisneria natans*	0, 0.1, 0.2, 0.5, 1 mg/L; 30d	20 to 300 μm	Reduced biomass, chlorophyll content and maximum quantum yields; affected structure and function of microbial communities in the phyllosphere and rhizosphere	(Chen et al., 2025)

Type	Species	Concentration and exposure time	Size	Reported biological responses	References
PS	*Spirodela polyrhiza*	0, 10^2, 10^3, 10^4, 10^5, and 10^6 particles/mL; 120h	50 and 500 nm	No effects on growth and chlorophyll production; 50 nm microplastics adsorbed on roots	(Dovidat et al., 2020)
PE	*Oscillatoria Princeps* and *Chlorella Pyrenoidosa*	100 mg/L; 5d	6.5 and 13μm	Growth inhibition; reduced chlorophyll a content and superoxide dismutase activity; changes in phycotoxin levels	(Zhao et al., 2024b)
PE	*Vallisneria natans*	10 mg/L; 28d	50 nm, 500 nm and 5 μm	Oxidative stress; metabolic changes; enhanced microbial metabolic activities	(Zhang et al., 2024c)
PE	*Salvinia auriculata*	2.7×10^8 and 8.1×10^8 particles/m^3; 28d	Average size: 35.46 ± 18.17 μm	Alterations in plant growth/development; reduced chlorophyll content; cell membrane damage; oxidative stress	(Gomes et al., 2023)
PVC	*Chlamydomonas reinhardtii*	0, 10, 20, 50, 100 and 200 mg/L; 96h	50 μm to 100 μm	Growth inhibition; Decrease of chlorophyll content; oxidative damage	(Wang et al., 2020b)
PVC	*Spirodela polyrhiza*	0, 10, 100 and 1000 mg/L; 7d	Average size: 3.87 ± 3.14 μm	Inhibition of adventitious root elongation ratio and leaf multiplication ratio; affected plant nutrient metabolism	(Wang et al., 2023b)
PET	*Lemna minor*	1136 ± 85, 224 ± 18, and 80 ± 4 particles/mL; 7d	0 to 500 μm	Inhibition of growth and photosynthesis	(Cui et al., 2023)
PLA	*Vallisneria natans*	0, 1%, 2% and 5% (w/w); 21d	-	No effect on growth; increased antioxidant enzyme activities	(Xiang et al., 2025)

Note: PLA, polylactic acid.

1.4.2.4 Effects of microplastics on multi-species, and ecosystem structure and function

Current studies have primarily focused on the effects of microplastics at the individual-organism level, revealing impacts on development, metabolism, and physiology. However, only a limited number of studies have examined how microplastics influence multiple interacting species within freshwater ecosystems. Organisms inhabiting the same ecosystem are interconnected through complex trophic interactions, energy transfer, and material exchange (Chen et al., 2025). Therefore, investigating the effects of microplastics on multi-species in ecosystems and elucidating the interactions among different trophic levels is essential for advancing our understanding of how microplastics affect ecosystem structure, function, and overall stability.

For example, Chen et al. (2025) reported that microplastics can disrupt the metabolic, autotrophic, and heterotrophic behaviours of *Vallisneria natans* and *Sinotaia quadrata* through their symbiotic interactions. Similarly, glitter particles were shown to affect freshwater ecosystems by inhibiting the growth of the duckweed *Lemna minor* and promoting the proliferation of the invasive snail *Potamopyrgus antipodarum* (Green et al., 2021).

At the ecosystem scale, Marchant et al. (2023) found that the addition of microplastics had minimal effects on the structure and functioning of semi-natural freshwater ecosystems in mesocosm experiments. PE microplastics have been shown to alter sediment microbial composition and metabolism, inhibit plant growth, and disrupt nutrient cycles in freshwater ecosystems (Yu et al., 2022). Ecosystem multifunctionality, a composite measure of ecosystem processes and services under multiple stressors, has been increasingly used to assess ecosystem-level responses to microplastic contamination (Wang et al., 2022). For example, high-concentration PE microplastics (5%) reduced ecosystem multifunctionality by suppressing enzyme activity and altering microbial community (Liu et al., 2024). Microplastic exposure can lead to biodiversity loss and disrupt the intricate network of ecological interactions, ultimately degrading ecosystem functions and services (Bostan et al., 2023).

Although many studies have documented the effects of microplastics on individual freshwater organisms, evidence from multi-species and ecosystem-level studies remains

limited (Huang et al., 2021; Marchant et al., 2023). Existing research that incorporates interacting species is sparse and often yields inconsistent conclusions regarding how microplastic effects propagate through trophic interactions and influence ecosystem structure and function. Moreover, assessments of ecosystem multifunctionality under microplastic stress have focused predominantly on terrestrial soils, with comparatively little attention given to freshwater sediments and benthic environments. This imbalance highlights a key knowledge gap in understanding how microplastics affect ecosystem processes and stability within freshwater sediment-based systems.

1.4.3 Influence of the physical and chemical properties of micro- and nanoplastics on their biological responses

Physicochemical properties of micro- and nanoplastics (such as chemical composition, sizes, shapes, functionalization, and surface charge) influence the mobility, transformation, and fate of microplastics in the freshwater environment and determine the bioavailability and subsequent biological effects of microplastics in freshwater organisms (Li et al., 2023c). Micro- and nanoplastics will undergo weathering processes in the environment, such as ultraviolet (UV) radiation, physical stress and microbial degradation, which have been shown to affect the characteristics and biological responses of micro- and nanoplastics (Duan et al., 2021).

1.4.3.1 Chemical composition

Each micro- and nanoplastic type may be represented by different polymer types as manufacturers aim to produce plastics with specific properties (e.g., flexibility, roughness, resistance, and durability) (Espí et al., 2006). Thus, different types of micro- and nanoplastics possess distinct physical and chemical properties, which can influence their environmental behaviour, fate, and biological effects in freshwater ecosystems (Lozano et al., 2021).

For example, it has been reported that interactions between microplastics and freshwater microalgae are strongly polymer-dependent: PP microplastics exhibited

stronger hetero-aggregation with algal cells, leading to pronounced changes in particle density and enhanced sedimentation, whereas high-density PE microplastics showed weaker aggregation and more limited density alteration (Lagarde et al., 2016). Another study (Zheng et al., 2021) showed that exposure to different microplastic types (PVC, PS and PE) induces distinct biological responses in *Microcystis aeruginosa*, including growth inhibition, altered toxin production, and oxidative stress. PVC, PS, and PE microplastics all inhibited the growth of *Microcystis aeruginosa*, while the impact of PVC microplastics on *M. aeruginosa* was more severe than the other two microplastics. Zimmermann et al. (2020) exposed *Daphnia magna* to different types of microplastics and found that PVC was the most toxic to reproduction and PLA induced highest mortality. Meanwhile, the densities of different types of microplastics are different, which makes their vertical distribution in aquatic ecosystems vary and affects their bioavailability.

1.4.3.2 Size

Size is one of the key factors influencing the bioaccumulation and biological responses of micro- and nanoplastics in freshwater organisms (Gu et al., 2020). In general, the adverse effect of micro- and nanoplastics on organisms increases with the decrease of particle size (Sjollema et al., 2016). For example, Wang et al. (2023a) found that smaller PE and PS microplastics inhibited *Chlorella vulgaris* colony formation. Similarly, 50 nm nanoplastics caused higher mortality in *Daphnia magna* compared to 200 and 500 nm particles (Frankel et al., 2020). In zebrafish, exposure to low-density PE microplastics of various sizes resulted in decreased hatching rates, increased heart rates, and tissue damage, with smaller particles producing more obvious effects (Sankar et al., 2025). Yao et al. (2024) showed that plant growth, chlorophyll, ROS and antioxidant enzyme content had a positive correlation with microplastic size.

1.4.3.3 Shape

Micro- and nanoplastics occur in various shapes, including films, fragments, fibres, and spheres, which strongly influence their environmental transport, bioavailability, and toxicological behaviour (chouchene et al., 2023). For example, polyester fibres caused

deformities in the carapaces and antennas of freshwater zooplankton *Ceriodaphnia dubia*, while PE beads did not induce significant adverse effects (Ziajahromi et al., 2017). In zebrafish, fibrous microplastics persisted longer in the gastrointestinal tract and induced more severe intestinal responses compared with spherical particles (Qiao et al., 2019a). Furthermore, micro- and nanoplastics with sharp or irregular edges exerted stronger inhibitory effects on the growth of freshwater plants than those with smooth surfaces, while fibrous microplastics were observed to entangle plant root systems, causing root shrinkage (Kalčíková et al., 2017; Roy et al., 2022). Overall, irregularly shaped micro- and nanoplastics tend to exhibit greater adverse impacts than spherical forms, likely due to their enhanced physical abrasiveness, prolonged retention time, and increased interaction with biological tissues.

1.4.3.4 Weathering and aging process

After being released into the environment, micro- and nanoplastics undergo weathering and aging processes under various environmental factors, such as physical abrasion, temperature fluctuations, UV radiation, and microbial degradation, which render their physicochemical properties increasingly complex (Burrows et al., 2024; Luo et al., 2025). The aging process is a multifactorial and dynamic process with long temporal scales. Therefore, researchers often simulate aging in the laboratory using artificial methods, including light irradiation, chemical oxidation, and mechanical abrasion (He et al., 2023) Among these, photoaging has been most widely applied to accelerate the aging process.

The aging process can induce alterations on surface roughness, specific surface area, oxygen-containing functional groups and adsorption affinity for pollutants of the micro- and nanoplastics, thereby affecting their bioavailability and biological effects in freshwater organisms (Li et al., 2025b). Many studies have demonstrated that the photoaging of micro- and nanoplastics can induce stronger oxidative stress and exert greater biological effects than pristine particles (Xu et al., 2024). For example, UV-aged PVC microplastics induced more growth inhibition and oxidative damage in the freshwater algae *Chlamydomonas reinhardtii* (Wang et al., 2020b). Similarly,

Michailidou et al. (2024) found that aged PE microplastics induced more adverse impacts than their pristine ones, activating antioxidant defence mechanisms in the freshwater fish *Perca fluviatilis*. Photoaging has also been shown to increase the biological effects of micro- and nanoplastics to zebrafish, causing growth inhibition, elevated oxidative stress, decreased hatching rates, increased mortality, and larval damage (Long et al., 2025; Zhang et al., 2022d).

However, some studies have reported contrary findings, suggesting that aging can sometimes mitigate micro- and nanoplastic effects. For example, UV radiation reduced the adverse effects of PS nanoplastics on the freshwater microalga *Scenedesmus obliquus*, likely due to an increase in particle aggregation and hydrodynamic size in the exposure medium (Giri & Mukherjee, 2021). Similarly, Ebbesen et al. (2024) observed negligible effects of aged microplastics on planktonic communities following long-term exposure to environmentally relevant concentrations. Overall, elucidating the processes and mechanisms of micro- and nanoplastic aging is essential for assessing their ecological risks in freshwater ecosystems.

1.5 Polystyrene (PS) micro- and nanoplastics in freshwater systems

Although the studies reviewed above provide important evidence on the biological responses of micro- and nanoplastics in freshwater organisms, their findings cannot be applied equally to all polymer types. Micro- and nanoplastics differ in polymer composition, density, size, shape, surface chemistry and weathering behaviour, all of which can influence environmental fate, bioavailability and biological effects (Li et al., 2023c). PS was selected as the focal polymer in this thesis for three main reasons. First, PS is environmentally relevant because PS particles have been detected in freshwater surface waters and sediments together with other common polymers. Second, PS is experimentally useful because commercially available PS particles can be obtained in defined sizes, including the 1 μm PS microplastics used in Chapter 2 and the 500 nm PS nanoplastics used in Chapter 3. Third, PS particles have been widely used in previous laboratory studies, which allows data standardization and a comparison of the results of our study with those of other studies on particle uptake, oxidative stress, gut microbiota

disturbance, metabolic alteration, reproductive responses and sediment-associated effects. The following section specifically reviews PS micro- and nanoplastics in freshwater systems, including their sources, entry pathways, transport, freshwater occurrence, transformation and stress-related or toxicity-related biological responses.

1.5.1 Sources and environmental entry pathways of polystyrene (PS) micro- and nanoplastics

PS is a widely used synthetic polymer produced through the polymerisation of styrene monomers and is commonly used in expanded foam materials, packaging, disposable food containers, insulation materials, toys, cup covers and other consumer products (Jalaudin Basha et al., 2023; Kik et al., 2020). The main sources of PS micro- and nanoplastics in freshwater systems are linked to the production, use, disposal and fragmentation of PS-containing materials. Larger PS debris can fragment into smaller PS microplastics and PS nanoplastics through ultraviolet radiation, mechanical abrasion, temperature fluctuation, chemical oxidation and biological colonisation (Kik et al., 2020; Xu et al., 2024). PS particles can enter freshwater systems through pathways similar to those described for microplastics, including wastewater treatment plant effluent, sewage sludge, landfill leachate, stormwater runoff, urban drainage, atmospheric deposition and inappropriate waste disposal (Eerkes-Medrano et al., 2015; Jalaudin Basha et al., 2023). Because PS is persistent and not readily biodegradable, discarded PS materials may accumulate in landfills, urban drainage systems, riverbanks, surface waters and sediments before or after fragmentation (Eerkes-Medrano et al., 2015; Jalaudin Basha et al., 2023).

1.5.2 Transport, freshwater abundance and sediment interaction of polystyrene (PS) micro- and nanoplastics

The transport and retention of PS particles in freshwater systems are controlled by particle density, size, shape, surface charge, aggregation behaviour, biofilm formation and hydrodynamic conditions (Eerkes-Medrano et al., 2015; Waldschläger and Schüttrumpf, 2020). PS has a density close to freshwater, approximately 1.05 g cm^{-3}, so PS particles

may remain suspended, aggregate, settle or become remobilised depending on particle size, water chemistry, turbulence, biofouling and interaction with mineral or organic matter (Fan et al., 2022; Waldschläger and Schüttrumpf, 2020). Biofilm formation and hetero-aggregation can increase the effective density of plastic particles and promote their downward transport into sediments, even when the original polymer density would favour suspension or slow settling (Rummel et al., 2017; Kooi et al., 2017).

As discussed in Section 1.3.3, freshwater monitoring studies have reported substantial spatial and methodological variability in microplastic abundance across surface waters and sediments (Koelmans et al., 2019; Wang et al., 2021b). Within this broader freshwater microplastic evidence, PS has been detected widely in surface waters and sediments. For example, PS has been reported together with PE and PP in surface waters from the Lower Yellow River, the Three Gorges Reservoir, the Rhine River and Garda Lake, and together with PE and PVC in the Wei River (Di and Wang, 2018; Ding et al., 2019; Han et al., 2020; Mani and Burkhardt-Holm, 2020; Sighicelli et al., 2018). PS has also been identified in freshwater sediments, including sediments from Tibetan Plateau rivers, the Wen-Rui Tang River and the Wei River, where it occurred together with other polymers such as PET, PA, PE, PP, PVC and PES (Ding et al., 2019; Jiang et al., 2019a; Zhang et al., 2020). These findings indicate that PS is present in both water-column and sediment compartments of freshwater systems, although its abundance varies among sites and is usually reported as part of mixed-polymer microplastic pollution.

The variability in reported PS occurrence partly reflects genuine environmental heterogeneity and partly reflects methodological differences among studies. Freshwater microplastic abundance is influenced by source intensity, hydrological transport, population density, wastewater discharge, land use, rainfall events and local retention processes (Horton et al., 2017; Wagner et al., 2014; Xia et al., 2020; Talbot and Chang, 2022). At the same time, reported polymer composition and abundance are affected by sampling strategy, mesh size, extraction method, digestion procedure, particle-size detection limit, polymer-identification technique and reporting unit (Eerkes-Medrano et al., 2015; Koelmans et al., 2019; Yang et al., 2021).

1.5.3 Transformation and weathering of polystyrene (PS) micro- and nanoplastics in freshwater ecosystems

As discussed in Section 1.3.4, microplastics in freshwater ecosystems undergo continuous physical, chemical and biological weathering, which can modify particle size, surface properties and density, thereby influencing transport behaviour, accumulation patterns and environmental persistence (Li et al., 2022a; Duan et al., 2021; Luo et al., 2025). These general transformation processes are also relevant to PS particles because PS debris released from packaging, foam materials and other consumer products is unlikely to remain pristine after entering freshwater environments (Kik et al., 2020; Jalaudin Basha et al., 2023). Instead, PS particles may undergo ultraviolet (UV) aging, mechanical abrasion, chemical oxidation and biofilm colonisation, each of which can modify particle properties and subsequent biological interactions (Kik et al., 2020; Xu et al., 2024).

Chemical transformation of PS is often associated with UV-driven photooxidation. UV exposure can promote polymer-chain scission, surface oxidation, surface roughening and the formation of oxygen-containing functional groups, including carbonyl- and hydroxyl-containing groups (He et al., 2023; Xu et al., 2024). These changes may alter PS particle hydrophobicity, surface charge, aggregation behaviour and affinity for dissolved organic matter or co-occurring contaminants (Duan et al., 2021; Xu et al., 2024).

Physical transformation of PS particles can occur through abrasion, collision and fragmentation during transport in rivers, sediments and shorelines. Mechanical weathering can generate cracks, rougher surfaces, irregular fragments and more heterogeneous particle-size distributions (Enfrin et al., 2019; Yan et al., 2023). Such physical changes may influence PS particle settling behaviour, sediment interaction, tissue contact and retention by organisms (Waldschläger and Schüttrumpf, 2020; Yan et al., 2023). This is particularly relevant for sediment-associated exposure because mechanically weathered particles may interact differently with mineral particles, organic matter, plant roots, benthic invertebrates and sediment microbial communities compared with smoother pristine particles (Yang et al., 2021; Yan et al., 2023).

Biological transformation further complicates the environmental behaviour of PS

particles. Once released into freshwater systems, PS particles can be colonised by microorganisms and incorporated into biofilms, forming part of the plastisphere (Zettler et al., 2013; Rummel et al., 2017). Biofilm colonisation can alter PS surface chemistry, increase aggregation, change effective particle density and affect sedimentation or remobilisation processes, and influence organismal exposure (Rummel et al., 2017; Kooi et al., 2017; Zettler et al., 2013).

Although weathering can modify PS particle properties, UV aging, mechanical weathering and biofilm colonisation affect particles through different mechanisms and may produce different biological consequences (Besseling et al., 2019; Xu et al., 2024; Yan et al., 2023). Therefore, aged or weathered PS particles should be interpreted according to the specific transformation pathway and measured particle properties.

1.5.4 Biological effects of polystyrene (PS) micro- and nanoplastics in freshwater organisms

Building on the general framework of biological responses reviewed in Section 1.4, this section focuses specifically on the effects of PS micro- and nanoplastics in freshwater organisms. PS particles have been widely used in laboratory ecotoxicology because they can be produced in controlled size ranges, are commercially available, and can be used to investigate particle uptake, tissue association and sublethal biological responses under defined exposure conditions (Lu et al., 2016; Pitt et al., 2018a; Kik et al., 2020).

Fish have been the most frequently used vertebrate models for assessing PS-related biological effects (Lu et al., 2016; Pitt et al., 2018a; Qiao et al., 2019a). In fish, the gastrointestinal tract is a primary interface between PS particles and the organism because ingested particles can be retained in the gut, contact the intestinal epithelium, interact with gut contents and alter resident microbial communities (Lei et al., 2018; Qiao et al., 2019b; Teng et al., 2022). In zebrafish, PS micro- and nanoplastic exposure has been associated with intestinal inflammation, altered gut barrier function, microbiota disturbance and metabolomic changes, indicating that gut responses are central to PS-induced biological disturbance (Qiao et al., 2019b; Teng et al., 2022). These intestinal responses may be stress-related when they involve changes in microbiota composition, inflammatory

signalling, mucus response or metabolite profiles, but they become stronger evidence of toxicity-related adverse outcomes when accompanied by severe histopathological damage, persistent barrier dysfunction, impaired feeding or reduced survival (van der Oost et al., 2003; Ankley et al., 2010; Marty et al., 2017).

The liver is another major target system for PS-related responses because it regulates detoxification, lipid metabolism, energy homeostasis and antioxidant defence in fish (Lu et al., 2016; Qiao et al., 2019b). PS microplastic exposure has been linked to hepatic oxidative stress, lipid-metabolism alteration and broader metabolic disturbance in zebrafish, while omics-based evidence suggests that PS microplastics can affect glycolipid metabolism, the tricarboxylic acid cycle, lipid catabolism, lipogenesis and energy homeostasis (Lu et al., 2016; Qiao et al., 2019b; Afsa et al., 2025). These findings suggest that PS particles may disturb metabolic regulation through interactions among intestinal exposure, microbial changes and liver metabolism.

Beyond gut and liver responses, PS particles may also affect broader regulatory axes in fish. The gut-liver axis provides a framework for linking intestinal microbiota, inflammatory signalling and hepatic metabolism, while the brain-intestine-microbiota axis links intestinal condition with neurotransmitter metabolism, behaviour and development (Tripathi et al., 2018; Tilg et al., 2022; Teng et al., 2022). Teng et al. (2022) showed that exposure to environmentally relevant concentrations of PS nanoplastics altered zebrafish gut microbiota, inflammatory responses, gut permeability and neurotransmitter-related metabolites, and was associated with reduced spontaneous movement, hatching rate and body length in F1 embryos. These findings indicate that PS nanoplastics can affect host-level regulation across gut, microbial, immune and neural pathways (Teng et al., 2022).

Reproduction and development related responses provide evidence of sublethal stress and potential fitness consequences. PS nanoplastics have been reported to affect developing zebrafish through tissue distribution, developmental alteration and behavioural endpoints, while parental exposure studies have shown that PS nanoplastics can be transferred from adult zebrafish to offspring (Pitt et al., 2018a; Pitt et al., 2018b; Teng et al., 2022). Evidence from fish models also suggests that PS microplastics may

disturb endocrine and reproductive pathways, as shown by altered hypothalamic-pituitary-gonadal signalling, steroidogenic pathways and gender-dependent reproductive outcomes in marine medaka (Wang et al., 2019). However, gender-specific responses of freshwater fish to PS particles remain less well characterised (Pitt et al., 2018a; Bhagat et al., 2020; Afsa et al., 2025).

PS micro- and nanoplastics can also influence non-fish freshwater organisms and sediment-associated components. In freshwater primary producers, PS microplastics have been shown to alter reactive oxygen species production, microcystin production and metabolomic profiles in *Microcystis aeruginosa*, and clear growth and photosynthesis inhibition were observed under the tested exposure conditions (Fan et al., 2022). In freshwater macrophytes, PS particles can adsorb to roots or plant surfaces and may affect growth, functional traits, photosynthesis-related endpoints and oxidative-stress responses, although responses vary among species, particle sizes and endpoints (Dovidat et al., 2020; Yu et al., 2020; Zhang et al., 2022b). In freshwater invertebrates, PS microplastics can negatively influence growth and emergence of freshwater sediment-dwelling invertebrate *Chironomus tepperi* (Ziajahromi et al., 2018). Sediment-associated effects are particularly relevant for PS particles because freshwater sediments can act as both sinks and biologically active interfaces for micro- and nanoplastics. Once PS particles enter sediments, they may interact with mineral particles, organic matter, pore water, benthic organisms, plant roots and sediment microbial communities (Rummel et al., 2017; Yang et al., 2021). These interactions may alter sediment microbial community composition, extracellular enzyme activities, respiration, dissolved organic carbon dynamics and nutrient cycling, although the direction and magnitude of these responses depend on polymer type, particle size, surface properties, concentration and exposure duration (Seeley et al., 2020; Chomiak et al., 2024; Kong et al., 2025). Although many studies have documented the effects of PS microplastics on individual freshwater organisms, evidence from multi-species and ecosystem-level studies remains limited.

1.6 Knowledge gaps and challenges

Despite substantial progress in understanding micro- and nanoplastic pollution,

significant knowledge gaps remain regarding their biological effects, stress-related responses and potential toxicity mechanisms in freshwater systems. Research is required which should address several key challenges.

(1) Gender-specific responses to PS micro- and nanoplastics in freshwater fish

Although many studies have examined the effects of micro- and nanoplastics on freshwater fish, gender-specific responses to PS particles remain largely overlooked. Understanding how males and females differentially respond to PS micro- and nanoplastic exposure, particularly in metabolic pathways, endocrine regulation, reproductive performance, and immune function, is essential for clarifying population-level impacts. Addressing these differences will improve predictions of population resilience and contribute to more accurate ecological risk assessments.

(2) Effects of PS particle transformation on biological responses

Micro- and nanoplastics in natural freshwater environments undergo continuous transformation through UV radiation, mechanical abrasion, chemical oxidation, and biofilm colonization. These processes alter particle surface chemistry, morphology, and the release of additives or sorbed pollutants. However, the mechanisms by which different transformation processes influences stress-related responses and potential toxicity outcomes induced by PS particles in freshwater organisms remain insufficiently understood. Clarifying these processes is crucial for evaluating ecological risks of PS micro- and nanoplastics in freshwater ecosystems.

(3) Multi-species and ecosystem-level impacts of PS microplastics

A majority of studies have focused on the effects of microplastics to single species, which offer valuable mechanistic insights but cannot fully capture community-level or ecosystem-level responses. Limited knowledge exists on how microplastics affect different functional groups of organisms, multi-species interactions, and ecosystem functions. Thus, there is a need to adopt multi-species or microcosm approaches to elucidate how microplastics influence ecosystem structure, function, and stability.

1.7 Research aims

The overarching aim of this thesis is to investigate the biological effects, stress-

related responses and potential toxicity-related adverse outcomes of polystyrene (PS) micro- and nanoplastics in freshwater organisms and ecosystem processes, assessing their potential risk to freshwater biota and ecosystem functions. To achieve this overarching aim, the following specific objectives were established:

(1) to investigate the gender-specific biological responses and potential reproduction-related adverse outcomes induced by pristine PS microplastics in adult zebrafish (*Danio rerio*), focusing on oxidative stress, immune-related indicators, gut microbiota, liver metabolism, stable isotope composition, sex hormone levels and egg production;

(2) to examine how UV aging process modifies the physicochemical properties and stress-related biological effects of PS nanoplastics in adult zebrafish by comparing responses to pristine and UV-aged PS nanoplastics, with particular emphasis on oxidative stress, immune-related indicators, gut microbiota disturbance and gut-liver axis associated metabolic responses; and

(3) to evaluate the effects of pristine and mechanically weathered PS microplastics in a laboratory-constructed freshwater-sediment microcosm comprising submerged macrophyte *Vallisneria spiralis*, freshwater snails *Lymnaea stagnalis*, and artificial sediments, and to determine how pristine and mechanically weathered PS microplastics influence organismal stress responses, sediment environmental properties, sediment enzyme activities, respiration and ecosystem multifunctionality.

1.8 Thesis structure and research methodology

1.8.1 Overview of research methodology

The research presented in this thesis takes a laboratory experimental approach to investigate the impacts of micro- and nanoplastics on freshwater organisms and freshwater-sediment systems. This approach was selected because micro- and nanoplastic effects are influenced by multiple interacting factors, including particle size, aging status, biological sex, exposure route and environmental compartment. Controlled laboratory experiments allow these factors to be examined under standardised conditions and enable stress-related biological responses and sediment-associated functional responses to be

interpreted more clearly.

Chapter 2 used adult zebrafish to examine gender-specific responses to pristine 1 μm PS microplastics. This model was selected because zebrafish are widely used in aquatic toxicology, have well-characterised reproductive biology and allow male and female responses to be analysed separately. Oxidative-stress biomarkers, skin mucus immune-related indicators, gut microbiota analysis, liver metabolomics, stable isotope analysis, sex hormone levels and egg production were measured to assess stress-related physiological, microbial and metabolic responses, as well as potential reproduction-related adverse outcomes.

Chapter 3 used adult zebrafish exposed to pristine and UV-aged 500 nm PS nanoplastics to test whether UV aging altered biological responses. UV aging was selected to represent a controlled photooxidative transformation process, and particle characterisation was performed using microscopy and surface-chemistry analysis. Skin mucus related indicators, oxidative stress, gut microbiota analysis, liver metabolomics and integrated biomarker response analysis were used to assess stress-related responses associated with gut-liver interactions.

Chapter 4 used a simplified freshwater-sediment microcosm containing artificial sediment, *V. spiralis* and *L. stagnalis* to examine responses to pristine and mechanically weathered PS microplastics. This design was chosen to move beyond single-organism exposure and assess interactions among particles, sediment properties, plants, snails and sediment-associated microbial processes. Plant pigments and oxidative-stress indicators, snail enzyme responses, sediment physicochemical properties, sediment enzyme activities, respiration and ecosystem multifunctionality were measured to assess organismal stress and sediment-associated functional responses. Because the system was simplified and sampled at a single terminal time point, the results are interpreted as controlled microcosm responses rather than direct predictions of natural freshwater ecosystem outcomes.

1.8.2 Thesis structure

Chapter 2 investigates stress-related biological responses and potential toxicity-

related adverse outcomes of PS microplastics in adult zebrafish, with particular emphasis on gender-specific differences (objective 1). Briefly, adult zebrafish were exposed to PS microplastics under laboratory conditions, and multiple biological endpoints were assessed, including skin mucus immune-related indicators, gut microbiota composition, oxidative stress biomarkers, liver metabolism, and reproductive performance.

Chapter 3 examines whether UV aging modifies stress-related responses to PS nanoplastics in adult zebrafish (objective 2). Briefly, adult zebrafish were exposed to pristine and UV-aged PS nanoplastics under controlled laboratory conditions. Stress-related responses were assessed through indicators associated with the gut-liver axis to elucidate underlying mechanisms.

Chapter 4 explores the effects of pristine and mechanically weathered PS microplastics using a laboratory-established freshwater-sediment microcosm comprising artificial sediment, the submerged macrophyte *V. spiralis* and the freshwater snail *L. stagnalis* (objective 3). Organismal stress responses, changes in water and sediment properties, and alterations in ecosystem multifunctionality were systematically evaluated.

Chapter 5 presents an integrative discussion of the overall findings in relation to the study objectives, summarises the major conclusions and offers recommendations for future research directions.

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Chapter 2. Female zebrafish are more affected than males under polystyrene microplastics exposure

2.1 Abstract

Microplastics are ubiquitous in freshwater and can be absorbed into fish skin and gills, accumulate in the gut, and be transported to other tissues, thus posing a risk to fish health. However, existing studies have insufficiently addressed whether adult female and male fish respond differently to polystyrene (PS) microplastic exposure across multiple biological levels, including mucus immunity, oxidative stress, gut microbiota, hepatic metabolism and reproduction. This limits the ability to identify gender-specific sensitivity and to link early stress responses with adverse reproductive outcomes. In this study, zebrafish were exposed to PS microplastics at 30 mg/L for 21 days. Multiple biological endpoints were measured, including PS microplastic accumulation in tissues, skin mucus related indicators, oxidative stress, gut microbiota, liver metabolomics, stable isotope composition, sex hormone level and egg production. Results showed that concentrations of PS microplastics were higher in gills than those in the gut. Minimal impact to immunoglobulin M level and lysozyme activity in mucus indicated PS microplastics did not cause obvious disruption of mucus immune indicators. Female zebrafish showed broader responses than males. Gut microbiota dysbiosis was induced, especially in females. Significant alterations in pathways associated with lipid and energy metabolism were observed in the liver of female fish. PS microplastics also induced sex steroid hormone disorder and reduced female egg production, possibly linked to the alteration of gut microbiota and hepatic metabolism. Combined, these results show that 1 μm PS microplastic exposure induced gender-specific stress-related biological responses and potential reproduction-related adverse outcomes in adult zebrafish, highlighting the importance of considering biological sex in freshwater microplastic ecotoxicology.

2.2 Introduction

Microplastics, as novel pollutants, are widely detected in water environments, posing a threat to organisms and ecosystems (Wang et al., 2022b). Detected microplastic concentrations vary across different water environments, and can even reach ~70 mg/L in wastewater treatment plants and ~ 0.10 g/L in surface water in Malaysia and Brazil (Li et al., 2020; Xu et al., 2021; Zhang et al., 2023). Concerns about microplastics in freshwater ecosystems have become higher profile (Bexeitova et al., 2024; Mishra et al., 2022). Within freshwater ecosystems, fish communities are important receptors and bioindicators of microplastic pollution because of their localized habitats and diverse traits (Parker et al., 2021). Much research in recent years has focused on the toxicity of microplastics to freshwater fish (Chen et al., 2022; Mak et al., 2019; Wang et al., 2022a). When fish are exposed to microplastics in freshwater, the main absorption pathways include oral, gill and skin routes (Bhagat et al., 2020). Microplastics may adhere to the skin surface due to sticky mucus found on fish and this skin mucus acts as the main non-specific defence mechanism (Huang et al., 2022; Karbalaei et al., 2021; Montenegro et al., 2020). Yang et al. (2020) found that co-exposure of polystyrene (PS) microplastics and 6:2 chlorinated polyfluoroalkyl ether sulfonate reduced lysozyme activity, providing evidence of inflammation.

Apart from absorption on the skin and gills, ingested microplastics mainly accumulate in the gut and may be transported to other tissues, including the liver and gonads (Kim et al., 2021). The gut is a target tissue for microplastic accumulation, leading to adverse effects on fish health (Bhuyan, 2022; Li et al., 2022; Lu et al., 2016). Qiao et al. (2019a) found microplastics induced gut microbiota dysbiosis, mucosal damage and inflammation in zebrafish gut. Dysbiosis of gut microbes is linked to gut inflammation and can indicate host metabolic disorders (Ganz et al., 2011; Zhang et al., 2010). The gut-liver axis provides an important conceptual framework for understanding the interconnectedness of these responses. This axis refers to the bidirectional relationship among the gut, gut microbiota and liver, through which microbial metabolites, inflammatory signals and gut-derived substances may influence hepatic metabolism and physiological homeostasis (Albillos et al., 2020). Therefore, microplastic-induced

disturbance in the gut may not remain a local intestinal response, but may also be associated with liver oxidative stress, metabolic alteration and broader organismal stress responses. The liver plays a vital role in maintaining biological energy homeostasis and health (Castegna et al., 2020; Luo et al., 2019). Studies have shown that PS microplastics can disturb metabolism in fish (Lu et al., 2016; Ye et al., 2021), and stable isotope analysis has recently been applied to investigate changes in metabolic pathways after stress exposure (Ek et al., 2015). Toxic exposure could influence $\delta^{15}N$ and $\delta^{13}C$ of organisms by affecting growth, metabolism, and element turnover (Ek et al., 2016; Ek et al., 2015; Karlson et al., 2018). However, current knowledge of the isotopic effects of microplastics on fish remains limited.

In addition to research on the effects of microplastics on gut microbiota and metabolism, studies on reproductive toxicity are emerging, indicating microplastics induced alteration in tissue formation of zebrafish gonads, female zebrafish fecundity rate and hormonal homeostasis (Gupta et al., 2023; Qiang & Cheng, 2021). Sun et al. (2024) demonstrated that 50 nm PS nanoplastics at 1 mg/L for 21 days disturbed the endocrine regulation pathways of the brain-pituitary-gonadal axis, inducing sex-specific reproductive toxicity to zebrafish. However, it remains unclear whether sex-specific reproductive effects are accompanied by broader gut and liver associated stress responses in adult fish exposed to PS microplastics.

While many studies have demonstrated the adverse effects of microplastics on fish health (Sulaiman et al., 2023), gender is usually a neglected factor when assessing their impacts, despite the fact that microplastics have gender-specific effects in other organisms (El Kholy & Al Naggar, 2023; Lee et al., 2021). Yang et al. (2022) found that aged PS microplastics induced gender-specific effects on mice liver lipid metabolism. Shen et al. (2024) also demonstrated that the response of gut microbiota and faecal metabolites of mice to the co-exposure of PS microplastics and lead was sex-specific: more faecal metabolites were influenced in female mice than in male mice. Differential responses of male and female organisms can cause variations in health impacts, affecting offspring and population stability (Teng et al., 2022; Wang et al., 2019b). However, a comprehensive understanding of the gender-specific impacts of microplastics on fish, encompassing

absorption, ingestion, and multi-tissue responses, is still lacking. This integrated design can help determine whether female and male fish differ only in specific reproductive endpoints or whether they show broader gender-dependent physiological and metabolic sensitivity. Such information is important for improving freshwater microplastic hazard assessment, because risk may be underestimated if biological sex and cross-tissue responses are not considered.

This study aimed to investigate the gender-specific stress-related biological responses and potential reproductive adverse outcomes induced by PS microplastics in zebrafish. Adult zebrafish were exposed to 30 mg/L PS microplastics (1 μm) for 21 days. Based on the conceptual distinction between organismal stress and organismal toxicity established in Chapter 1, this chapter distinguishes stress-related responses from potential toxicity-related adverse outcomes. The stress-related hypothesis was that exposure to 1 μm PS microplastics would induce gender-specific physiological, microbial and metabolic responses in adult zebrafish. This hypothesis was tested using particle uptake analysis, oxidative stress biomarkers, skin mucus immune-related indicators, gut microbiota, liver metabolomics and stable isotope analysis. The toxicity-related hypothesis was that PS microplastic exposure may alter reproduction-related endpoints. This was assessed using sex hormone measurements and egg production. Because fertilisation success, offspring viability, recovery and long-term reproductive performance were not measured, reproductive changes were interpreted as potential adverse outcomes.

2.3 Materials and methods

The methodological design of this chapter was structured to evaluate PS microplastic exposure, stress-related responses and reproduction-related outcomes in adult female and male zebrafish. First, fluorescent PS microplastics were used to assess particle uptake and tissue association, while non-fluorescent virgin PS microplastics were used for the sublethal biological response experiment. Second, oxidative stress biomarkers, mucus immune-related indicators, gut microbiota profiling, liver metabolomics and stable isotope analysis were used to assess stress-related physiological, microbial and metabolic

responses. Third, sex hormone levels and egg production were measured to assess reproduction-related outcomes after PS microplastic exposure. Biomarker, microbiome and metabolomic endpoints are interpreted as stress-related biological responses, while reproduction-related endpoints are interpreted as potential adverse outcomes.

2.3.1 Microplastics

As one of the most abundant polymers in the environment, PS microplastics were chosen (Wang et al., 2023). PS microplastics with a diameter of 1 μm were purchased from BaseLine ChromTech Research Centre (Tianjin, China). Fluorescent microplastics (excitation wavelengths, 488 nm; emission wavelengths, 518 nm) were used in the uptake and accumulation test whereas virgin microplastics were used for the toxicity test. The 1 μm particle size was selected because it represents the lower boundary of the microplastic size class and therefore provides a high-bioavailability small microplastic exposure scenario. This size is suitable for controlled laboratory exposure and fluorescence-based tissue association analysis.

2.3.2 Zebrafish maintenance and experimental setup

Healthy wild-type adult zebrafish (AB strain), aged 5 months, were purchased from the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China). The zebrafish were kept in ultraviolet-sterilized and aerated water at 24 ± 1 °C under a 14 h light/10 h dark cycle and were fed brine shrimp twice daily. As shown in Fig. 2.1, for the accumulation test, acclimated zebrafish of both genders were randomly placed into 16 glass tanks, assigned to control and fluorescent PS microplastics (30 mg/L) treatment groups. 30 mg/L is a high-exposure laboratory concentration to provide us with insights into mechanistic responses. Assuming spherical PS particles with a diameter of 1 μm and a PS density of 1.05 g/cm^3, 30 mg/L corresponds to approximately 5.5×10^7 particles/mL. Each treatment had four replicates containing 10 zebrafish, with female and male fish exposed separately. Female and male zebrafish were housed separately during exposure to allow gender-specific biological responses to be attributed to each sex without

confounding by mating behaviour, reproductive interactions or unequal exposure caused by gender-related behavioural differences. This design was necessary because the main objective of the chapter was to compare female and male physiological, microbial and metabolic responses to PS microplastics. However, single-sex housing may have altered baseline reproductive condition or spawning readiness. After 21 days of exposure, fish were rinsed with ultra-pure water, euthanized using a 100 mg/L MS-222 (tricaine) solution, and the gut and gill tissues dissected for concentration analysis.

For the biological response experiment, acclimated female and male zebrafish were exposed to 0 (control) and 30 mg/L PS microplastics separately for 21 days. Each group contained four replicate glass tanks: approximately 30 zebrafish were included in each tank randomly, and female and male fish were exposed separately. A 21-day exposure period was selected as a sub-chronic exposure window for adult zebrafish, which is commonly used in zebrafish microplastic toxicity studies. Because it is long enough to allow particle association, physiological stress responses, microbiota changes and reproductive responses to emerge, while limiting prolonged husbandry-related confounding factors such as ageing, tank-density changes and cumulative handling stress.

After exposure, the fish underwent a 24-hour fasting period before sampling and rinsing with ultra-pure water. Fish were euthanized using a 100 mg/L MS-222 (Tricaine) solution and the gut and liver were dissected for further analysis. Biological sampling was conducted at the end of the 21-day exposure to provide an integrated endpoint assessment of accumulated responses after sub-chronic exposure. This design allowed direct comparison among particle accumulation, oxidative stress, gut microbiota, liver metabolomics, stable isotopes and reproductive endpoints at the same exposure stage. However, a single terminal sampling point cannot capture the temporal dynamics of stress initiation, adaptation or recovery during exposure. Therefore, the mechanistic interpretation is limited to endpoint associations, and time-series sampling would be needed to distinguish early transient stress responses from sustained toxic effects.

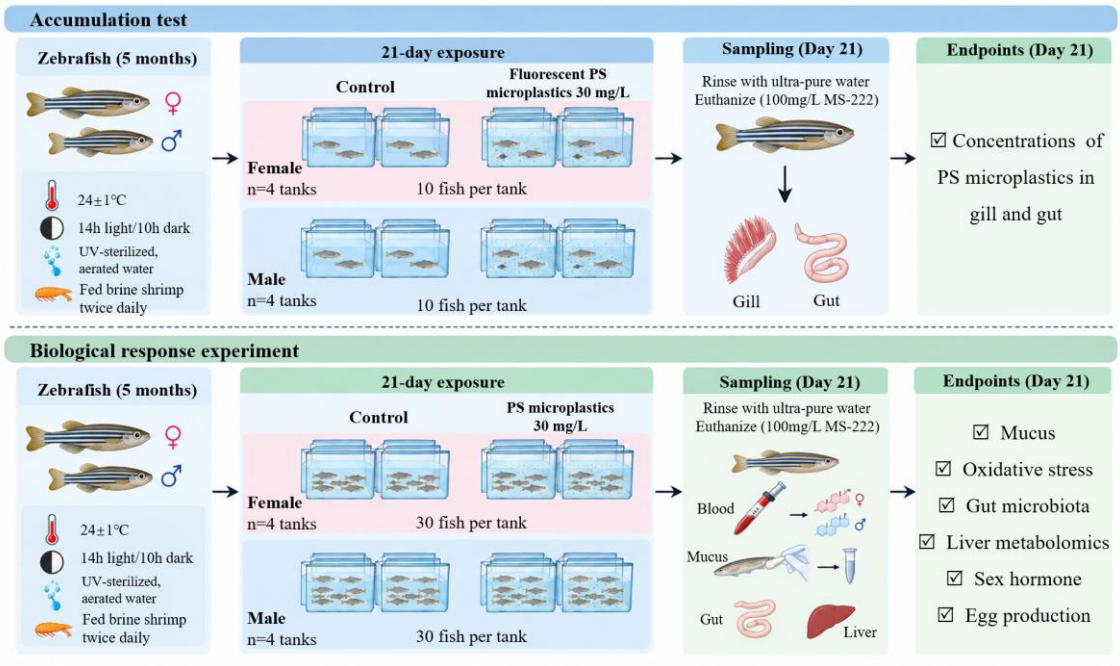


Figure 2.1. Schematic overview of the polystyrene (PS) microplastic exposure design and endpoint measurements in adult zebrafish.

2.3.3 Uptake and accumulation of polystyrene (PS) microplastics in fish tissues

Uptake and accumulation analysis was conducted to determine whether PS microplastics were associated with external and internal tissues after exposure. The gill and gut were selected to represent external and internal tissues, respectively. The gill was considered an external tissue because it is directly exposed to the surrounding water and can act as an initial interface for particle contact and uptake. The gut was considered an internal tissue because it is the primary site of particle ingestion, retention and potential translocation after exposure. According to methods outlined by previous studies (Dehaut et al., 2016; Lu et al., 2016; Zou et al., 2019), the zebrafish tissues (the gill and gut) were homogenized and then digested in KOH (100g/L) at 60 ℃ for 24h. The fluorescence intensity of fish samples was assessed using a microplate reader (Tecan Inifinite M200). The content of microplastics in zebrafish tissues was calculated using a standard curve. The standard curve was generated using serial dilutions of fluorescent PS microplastic suspensions with known concentrations. The background fluorescence of tissues from unexposed fish was measured after the same homogenisation and digestion procedure and

subtracted from the fluorescence intensity of exposed samples. To account for fluorescence loss or signal attenuation during tissue digestion, a recovery correction was performed. Known concentrations of fluorescent PS microplastics were added to digested tissues from unexposed fish and processed using the same KOH digestion and fluorescence measurement procedures as the experimental samples. The fluorescence recovery ratio was calculated by comparing the fluorescence signal of PS microplastics recovered from the digested tissue matrix with the fluorescence signal of the same PS microplastic concentration in the standard suspension. The apparent PS microplastic concentration calculated from the standard curve was then corrected using this recovery ratio. The corrected PS microplastic concentration was used to estimate the final PS microplastic content in zebrafish tissues.

2.3.4 Mucus determination

Skin mucus was analysed to assess external mucosal immune responses to PS microplastic exposure. Fish mucus represents an important first-line defence barrier in direct contact with the aquatic environment. Immunoglobulin M (IgM) and lysozyme (LZM) were selected as immune-related indicators because they reflect humoral immune response and antimicrobial defence at the mucosal surface (Yang et al., 2020). Mucus was collected using a non-lethal rubbing method after anaesthesia and washing, and the collected supernatant was used for commercial kit-based quantification. The skin mucus indicators were analysed according to Wang et al. (2019a). Briefly, 20 zebrafish from each treatment were anaesthetized, washed and transferred to a sterile polyethylene bag of 5 mL NaCl (100 mM). Fish were then gently rubbed back and forth on the bag for 2-3 minutes to collect the mucus. The collected samples were centrifuged (12000 rpm, 30 min, 4 ℃) and the supernatant was filtered using a 0.22 μm filter for further analysis. IgM and LZM of the mucus were measured using the Immunoglobulin M Assay Kit and lysozyme assay kit (Jiancheng, Nanjing, China) based on turbidity assays.

2.3.5 Oxidative stress of zebrafish

To evaluate the degree of oxidative stress in zebrafish liver, superoxide dismutase (SOD) activity and malondialdehyde (MDA) content were measured. Liver tissue homogenates (10%) were prepared with sterilized PBS at 4 ℃ and then centrifuged (12000 rpm, 10 min, 4 ℃) (Li et al., 2021). Commercial kits (superoxide dismutase assay kit and malondialdehyde assay kit) (Jiancheng, Nanjing, China) were used to measure the SOD activity and MDA content in collected supernatants.

2.3.6 16S rRNA gene sequencing analysis of gut microbiota

Gut microbiota analysis was performed to determine whether PS microplastic exposure altered gut microbial community composition and predicted microbial functions. The gut samples were collected and kept at -80 ℃ before further analysis. The CTAB/SDS method was used to extract the total genome DNA in samples. 16S rRNA genes in distinct regions (16S V3-V4) were amplified with specific primer and barcodes. An equal volume of 1X loading buffer (contained SYBR green) was mixed with PCR products and electrophoresis was performed on 2% agarose gel for DNA detection. The PCR products were mixed in equal proportions, and then Universal DNA PCR Purification Kit (TianGen, China, Catalog #: DP214) was used to purify the mixed PCR products. Following the manufacturer's recommendations, sequencing libraries were generated with NEB Next Ultra DNA Library Prep Kit for Illumina (NEB, USA, Catalog #: E7370L). The library quality was assessed on the Agilent 5400 system (Agilent, USA) and quantified by real-time PCR (1.5nM). Finally, the library was sequenced on an Illumina NovaSeq platform and 250 bp paired-end reads were generated. The raw reads were merged using FLASH (V1.2.7) and then filtered to remove low-quality reads and chimera sequences using QIIME (v1.9.1) and UCHIME. The effective tags were then clustered by UPARSE to Operational Taxonomic Units (OTUs) according to 97% similarity. For each representative sequence, the Silva Database was used based on Mothur algorithm to annotate taxonomic information and count the community composition of classification level (e.g., phylum and genus). Alpha diversity and beta diversity were calculated with QIIME. PCoA analysis was conducted based on Weighted

Unifrac distance and Unweighted Unifrac distance, and the principal coordinate combination with the highest contribution rate was selected for graphical display. Samples with high community structure similarity tend to cluster together, while samples with significant community differences will be far apart. Gut microbiota functions were predicted using Tax4Fun, and pathway information were performed on Kyoto Encyclopedia of Genes and Genomes (KEGG) database.The details of extraction of genome DNA, amplicon generation, PCR products quantification and qualification, library preparation and sequencing, and data analysis are listed in Appendix I. The sequencing data were deposited in the NCBI database (BioProject accession: PRJNA1116557). For gut microbiota analysis, four biological replicates were included in each group, except for the male 1μm PS microplastic group, in which three biological replicates were retained because one sample failed to meet sequencing quality-control criteria.

2.3.7 Liver metabolomics

Liver metabolomics was conducted to assess whether PS microplastic exposure altered hepatic metabolic regulation. Liver samples were collected and kept at -80 ℃ before further analysis. For metabolomics analysis, samples were extracted and detected by LC-MS. Mass spectrometric detection of metabolites was performed on Orbitrap Exploris 120 (Thermo Fisher Scientific, USA) with ESI ion source. Simultaneous MS1 and MS/MS (Full MS-ddMS2 mode, data-dependent MS/MS) acquisition was used. The parameters were as follows: sheath gas pressure, 30 arb; aux gas flow, 10 arb; spray voltage, 3.50 kV and -2.50 kV for ESI (+) and ESI (-), respectively; capillary temperature, 325 ℃; MS1 range, m/z 100-1000; MS1 resolving power, 60000 FWHM; number of data dependent scans per cycle, 4; MS/MS resolving power, 15000 FWHM; normalized collision energy, 30%; dynamic exclusion time, automatic.

The raw data were firstly converted to mzXML format by MSConvert in the ProteoWizard software package (v3.0.8789) and processed using XCMS for feature detection, retention time correction and alignment. The metabolites were identified by accuracy mass (< 30 ppm) and MS/MS data which were matched with HMDB, massbank,

LipidMaps, mzcloud and KEGG. The robust LOESS signal correction (QC-RLSC) was applied for data normalization to correct for any systematic bias. After normalization, only ion peaks with relative standard deviations (RSDs) less than 30 % in QC were kept to ensure proper metabolite identification. The Ropls software was used for all multivariate data analyses and modeling. Data were mean-centered using scaling. Models were built on principal component analysis (PCA), orthogonal partial least-square discriminant analysis (PLS-DA) and partial least-square discriminant analysis (OPLS-DA). The metabolic profiles could be visualized as a score plot, where each point represents a sample. The corresponding loading plot and S-plot were generated to provide information on the metabolites that influence clustering of the samples. All of the models evaluated were tested for over fitting with methods of permutation tests. The descriptive performance of the models was determined by R2X (cumulative) (perfect model: R2X (cum) = 1) and R2Y (cumulative) (perfect model: R2Y (cum) = 1) values while their prediction performance was measured by Q2 (cumulative) (perfect model: Q2 (cum) = 1) and a permutation test. The permuted model should not be able to predict classes: R2 and Q2 values at the Y-axis intercept must be lower than those of Q2 and the R2 of the non-permuted model. OPLS-DA allowed the determination of discriminating metabolites using the variable importance on projection (VIP). The variable importance projection (VIP) produced by OPLS-DA fold change (FC), was applied to discover the contributable-variable for classification. Finally, P values < 0.05 and VIP values > 1 were considered to be statistically significant metabolites.

The identified metabolites in metabolomics were then mapped to the KEGG pathway for biological interpretation of higher-level systemic functions. Details on sample extraction, LC-MS conditions and data analysis are provided in Appendix I.

2.3.8 Stable isotope analysis

Stable carbon and nitrogen isotope ratios were measured to provide an integrated indicator of whole-body nutrient assimilation and metabolic status after PS microplastic exposure. $\delta^{13}C$ can reflect changes in carbon source use, carbon turnover and energy

metabolism, whereas $\delta^{15}N$ is sensitive to nitrogen turnover, amino acid metabolism and protein balance (Ek et al., 2015). In this experiment, all zebrafish were maintained under the exposure conditions, so differences in $\delta^{13}C$ and $\delta^{15}N$ were interpreted as treatment-related physiological or metabolic shifts. Stable isotope analysis was therefore used as a complementary whole-organism indicator linking gut-associated disturbance, hepatic metabolism and reproductive performance. After freeze-drying (BIOSAFER-18A), zebrafish samples were milled for 1 minute (Retsch MM301) to obtain powder (< 100 μm). The stable isotope composition of samples was analysed using isotope ratio mass spectrometry (IRMS, Delta V Advantage, USA). The abundance of ^{13}C and ^{15}N was calculated using Eq. (2.1):

$$\delta X\ (‰) = ((R_{sample} - R_{reference})/R_{reference} \times 1000‰ \quad (2.1)$$

where R_{sample} is the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratio of the sample, and $R_{reference}$ is the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratio of the reference.

2.3.9 Impact of polystyrene (PS) microplastics on sex hormone levels and reproduction of zebrafish

Sex hormone levels and egg production were measured to assess whether PS microplastic exposure was associated with reproduction-related outcomes. 17β-oestradiol (E2) and testosterone (T) were selected because they are key steroid hormones involved in reproductive regulation. Egg production was assessed after controlled post-exposure pairing to determine whether exposure of females, males or both sexes influenced spawning output. Concentrations of E2 and T were analysed by using an oestradiol 2 assay kit and a testosterone assay kit (Jiancheng, Nanjing, China), following the manufacturer's instructions. In brief, zebrafish blood was collected and centrifuged (3000 rpm, 5 min, 4 °C) and the supernatants used for hormone concentration analysis (Pedroso et al., 2012). For reproduction, exposed or control females and males were subsequently paired in defined combinations. The spawning groups are control female × control male (♀ck × ♂ck), control female × PS microplastic exposed male (♀ck × ♂1μm), PS microplastic exposed female × control male (♀1μm × ♂ck), PS microplastic exposed female × PS microplastic

exposed male (♀1μm × ♂1μm). Here, ck represents the control group, 1μm represents exposure to 1 μm PS microplastics, ♀ represents female fish, and ♂ represents male fish. Egg production was measured as the number of eggs spawned by each post-exposure female-male pairing. Male zebrafish do not produce eggs; male exposure status was included to evaluate whether exposed males influenced spawning output during post-exposure pairing. After removing spawning zebrafish, the eggs spawned in each group were collected using a Pasteur pipette and counted manually. The egg production results should be interpreted as reproductive output after controlled post-exposure pairing, rather than as continuous reproductive performance during the full 21-day exposure.

2.3.10 Data analysis

The data are presented as mean ± standard deviation. Normality was assessed using the Shapiro-Wilk test, and homogeneity of variance was assessed using Levene's test. For comparisons involving two groups, normally distributed data with homogeneous variance were analysed using one-way analysis of variance (ANOVA), which is equivalent to an independent-samples t-test. If variance homogeneity was not met, Welch's t-test was used. For comparisons involving more than two groups, normally distributed data with homogeneous variance were analysed using one-way ANOVA followed by Tukey's post hoc test. If the data were normally distributed but variance homogeneity was not met, Welch ANOVA followed by Games-Howell post hoc test was used. A significance threshold of $p < 0.05$ was applied. Exact p-values for the statistical comparisons shown in the Chapter 2 figures are provided in Table S2.2. Because female and male zebrafish were housed, exposed and sampled separately, this study did not formally test sex × treatment interactions using a two-way ANOVA. Instead, within-sex comparisons were used to avoid confounding from baseline differences between females and males and to identify treatment-related changes relative to sex-specific controls.

2.4 Results and discussion

2.4.1 Effect of polystyrene (PS) microplastics on the physiochemical properties of zebrafish

2.4.1.1 Accumulation of polystyrene (PS) microplastics in zebrafish tissues and mucus analysis

PS microplastics accumulated in the gills and guts of fish (Fig. 2.2A). The concentration of PS microplastics in gills was higher than that in the gut for both genders. No significant difference was observed in immunoglobulin M (IgM) level and lysozyme (LZM) activity of zebrafish mucus in both genders compared to controls (Fig. 2.2B and C).

Previous studies have shown that the gills and guts are important tissues for the uptake and accumulation of microplastics, as they have a large surface area and are involved in nutrient absorption and immune defence (Batel et al., 2018). The accumulation concentration in different tissues depends on various factors, such as exposure concentration, time, and particle size of microplastics (Ding et al., 2018). Once PS microplastics enter the gut, there is the potential for them to result in adverse effects as well as translocation to other tissues via the circulatory system (Mark et al., 2008). As with our study, Yang et al. (2020) also found that PS microplastics did not influence LZM activity and IgM level in larval zebrafish. This suggests no significant skin mucus immune disruption after exposure to PS microplastics and that the biological responses to PS microplastics were related to ingestion into the gut instead of interaction with skin mucus.

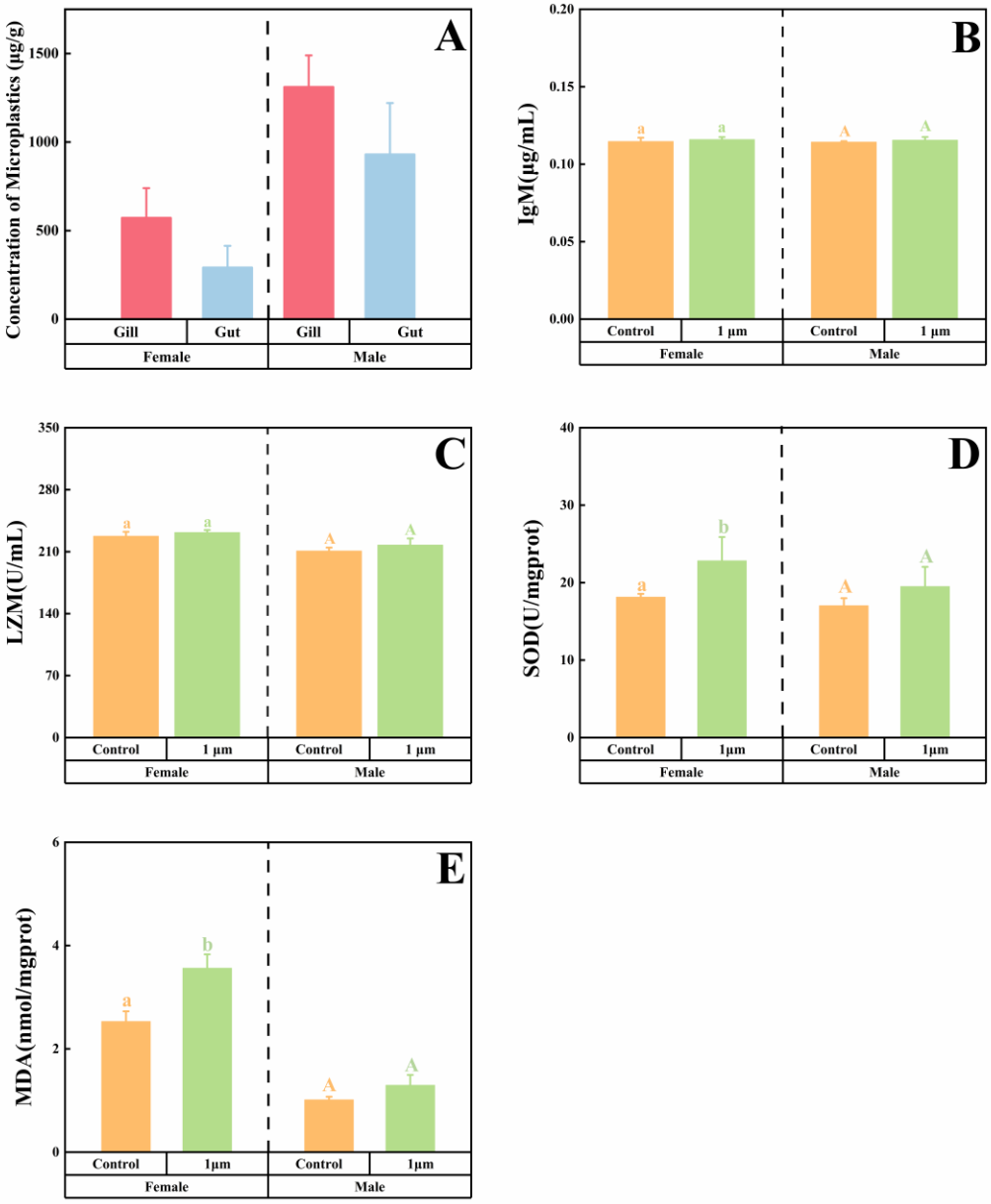


Figure 2.2. Effects of polystyrene (PS) microplastics exposure on microplastics uptake, skin mucus and oxidative stress of zebrafish. (A) Accumulation of PS microplastics in the gill and gut of zebrafish after exposure for 21 days. (B) Changes in immunoglobulin M (IgM) of zebrafish skin mucus after exposure to PS microplastics; (C) Lysozyme (LZM); (D) Superoxide dismutase (SOD) activities of zebrafish after exposure to PS microplastics; (E) Malondialdehyde (MDA). Data represent mean ± SD (n = 4). Different letters indicate significant differences between treatments ($p < 0.05$).

2.4.1.2 Hepatic oxidative stress

The female fish treatment showed significantly increased SOD activity and MDA content in the liver compared to the controls, while there was no such difference for male zebrafish (Fig. 2.2D and E).

The PS microplastic-induced oxidative stress in female zebrafish that we observed is similar to that found in earlier studies (Jeong et al., 2017; Qiao et al., 2019a). The increased SOD activity could reflect membrane damage in the liver, while as the indicator of cell membrane lipid peroxidation damage (Uchiyama & Mihara, 1978), increased MDA content might lead to ROS generation and apoptosis of hepatocytes.

2.4.2 Effects of polystyrene (PS) microplastics on zebrafish gut microbiota composition and function

2.4.2.1 Gut microbiota composition

The Shannon index reflects both richness and evenness of microbial communities. The Simpson index gives greater weight to dominant taxa and is commonly used to assess community evenness or dominance. Both indices were used to provide complementary information on overall alpha diversity and dominance structure. The Shannon and Simpson indices of gut microbiota increased significantly after exposure to PS microplastics in both genders (Table 2.1). There was also a large number of unique OTUs in female and male zebrafish when exposed to PS microplastics (Fig. 2.3A and B). The PCoA assessment (Fig. 2.3C and D) showed that the female group deviated from the control group, whereas this was not the case in the male group. Results indicated that PS microplastics increased the alpha diversity in both genders and caused changes in the beta-diversity of gut fauna in females. The increased Shannon and Simpson diversity indices indicated that PS microplastics enhanced the evenness of the gut microbial community (Feng et al., 2021), indicating that exposed zebrafish were more vulnerable to the invasion and infection of opportunistic pathogens and the niche of dominant bacteria was also suppressed (Hu et al., 2022).

In female zebrafish, as shown in Fig. 2.3E and 2.4A, Fusobacteria were dominant (over 70%) in the control while Proteobacteria were most abundant (over 50%) in the

1μm PS microplastics treatment. The relative abundance of Proteobacteria, Firmicutes and Bacteroidota increased in the 1μm PS microplastics treatment compared to the control group whereas the abundance of Fusobacteria decreased. In male zebrafish, the most abundant phylum was Proteobacteria (Fig. 2.3E and 2.4B) but the exposure to PS microplastics led to a lower relative abundance of Fusobacteria and Proteobacteria while the abundance of Actinobacteria and Firmicutes was greater. At genus level, results showed that, in female fish, PS microplastics decreased the content of *Cetobacterium*, whereas the relative abundance of *Pseudomonas*, *Aeromonas* and *Vibrio* increased (Fig. 2.4C). Meanwhile, in the male fish gut, PS microplastics decreased the abundance of *Cetobacterium*, *Plesiomonas*, *Aeromonas* and *Vibrio* (Fig. 2.4D). Apart from phylum and genus level, in the female microplastics exposure group, the abundance of the classes Gammaproteobacteria and Clostridia, the order Pseudomonadales, Lachnospirales and Bacteroidales, and the families Pseudomonadaceae and Lachnospiraceae were higher compared to the control (Fig. S2.1). In the male microplastics exposure group, the abundance of the class Bacteroidia and the unidentified Actinobacteria, the orders Pseudomonadales, Bacteroidales and Burkholderiales, and the family Pseudomonadaceae were greater than in the male control group (Fig. S2.2).

Table 2.1. Shannon and Simpson alpha diversity index of zebrafish gut microbiota after PS microplastics exposure.

Index	Shannon	Simpson
F. cont.	2.514 ± 0.771	0.559 ± 0.123
F. 1μm	4.663 ± 0.899*	0.872 ± 0.096*
M. cont.	4.508 ± 0.966	0.857 ± 0.062
M. 1μm	8.222 ± 0.282*	0.987 ± 0.002*

Note: F.Cont. represents the female control group, F.1μm represents the female PS microplastic-exposed group, M.Cont. represents the male control group, and M.1μm represents the male PS microplastic-exposed group. * indicates significant differences between treatments ($p < 0.05$).

Several studies have demonstrated that microplastics accumulation can alter gut microbiota composition in fish (Feng et al., 2021; Qiao et al., 2019a; Wan et al., 2019).

This is important because the dysbiosis of fish gut microbiota composition can lead to the development of many diseases in hosts, including obesity and diabetes (Snedeker & Hay, 2012). At phylum level, previous research found that gut inflammation was related to microbiota disorder and inflammatory-induced oxidation could promote the growth of Proteobacteria, which are able to cope with this adverse host environment (Huang et al., 2020). As Bacteroidetes and Firmicutes are related to lean body weight and fat absorption (Sheng et al., 2018; Turnbaugh et al., 2009; Wan et al., 2019), the variation of Bacteroidetes and Firmicutes abundance that we identified might influence the energy and lipid metabolism of zebrafish. At genus level, the decreased abundance of *Cetobacterium* (as a beneficial bacterium that can protect the host from pathogens (Gu et al., 2021)) and the enriched pathogens (including *Aeromonas* and *Pseudomonas* (Chandrarathna et al., 2018; Janda & Abbott, 2010; Oliver et al., 2000)) in female PS microplastic treatments suggested that female zebrafish gut function and health was influenced by PS microplastics.

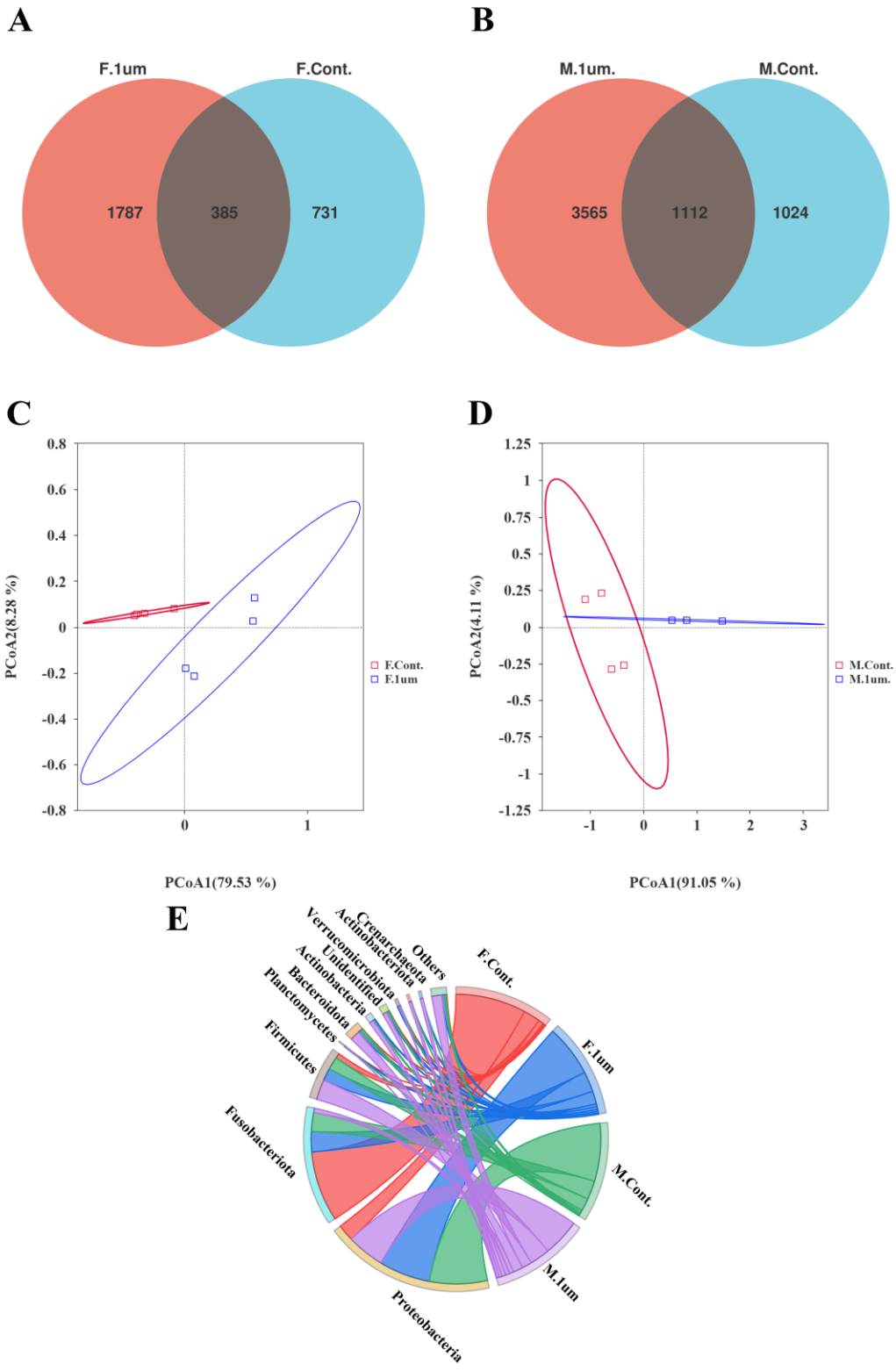


Figure 2.3. Assessment of difference or similarity of microbiota composition among different treatment groups after exposure to 1μm polystyrene (PS) microplastics for 21 days. Venn diagram of bacterial OTUs unique within the gut microbiota of zebrafish after exposure to PS microplastics. A, female groups; B, male groups. Principal coordinate

analysis (PCoA) of the gut microbiota beta-diversity of the control and PS microplastics treated groups, each point represents one gut microbiota sample, and greater distance between points indicates greater dissimilarity in microbial community structure, C, female groups; D, male groups. E, the chord diagram of gut microbiota abundance at phylum level after PS microplastics exposure. F.Cont. represents the female control group, F.1μm represents the female PS microplastic exposed group, M.Cont. represents the male control group, and M.1μm represents the male PS microplastic exposed group. For gut microbiota analysis, n = 4 for all groups except the male 1 μm PS microplastic group, for which n = 3 because one sample failed to meet sequencing quality-control criteria.

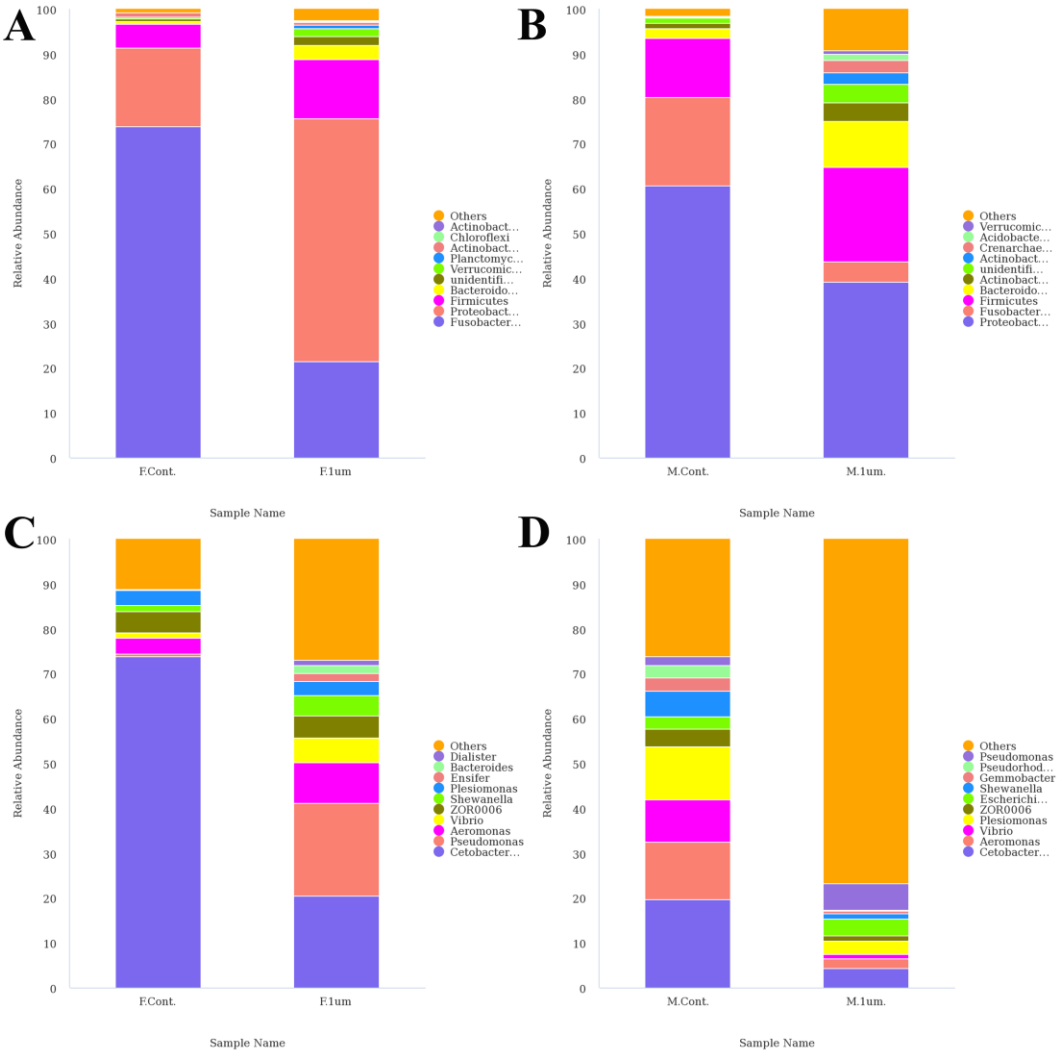


Figure 2.4. The relative abundance of gut microbiota at phylum level after polystyrene (PS) microplastics exposure: (A) female groups; (B) male groups. The relative abundance of gut microbiota at genus level after PS microplastics exposure: (C) female groups; (D)

male groups. F.Cont. represents the female control group, F.1μm represents the female PS microplastic exposed group, M.Cont. represents the male control group, and M.1μm represents the male PS microplastic exposed group. Relative abundance is presented as the percentage contribution of each taxon to the total bacterial community. Taxa with low relative abundance are grouped as "Others".

2.4.2.2 Predicted function of gut microbiota

Changes in the gut microbiota of zebrafish were accompanied by significant changes in the predicted functions of the microbiota, particularly in females (Fig. 2.5-2.6 and S2.3-S2.4). At level 1, predicted pathways of gut microbiota including metabolism, genetic information processing and organismal systems showed under-representation. Conversely, pathways such as environmental information processing, cellular processes and human diseases showed significant enrichment in females in the treatments compared to the control. In the male group, only metabolism and environmental information processing were significantly affected by PS microplastics.

According to the level 2 and level 3 functional prediction map of the metabolic pathway (Fig. 2.6 and S2.3-S2.4), more metabolic pathways in female fish were significantly affected than those in male fish after PS microplastics exposure. In the female zebrafish group, the pathways related to nutrient transport and metabolism, as well as energy production and conversion (including amino acid, carbohydrate, nucleotide, cofactors and vitamins, and energy metabolism) were significantly influenced by PS microplastics, indicating impacts on the basic metabolism of female zebrafish.

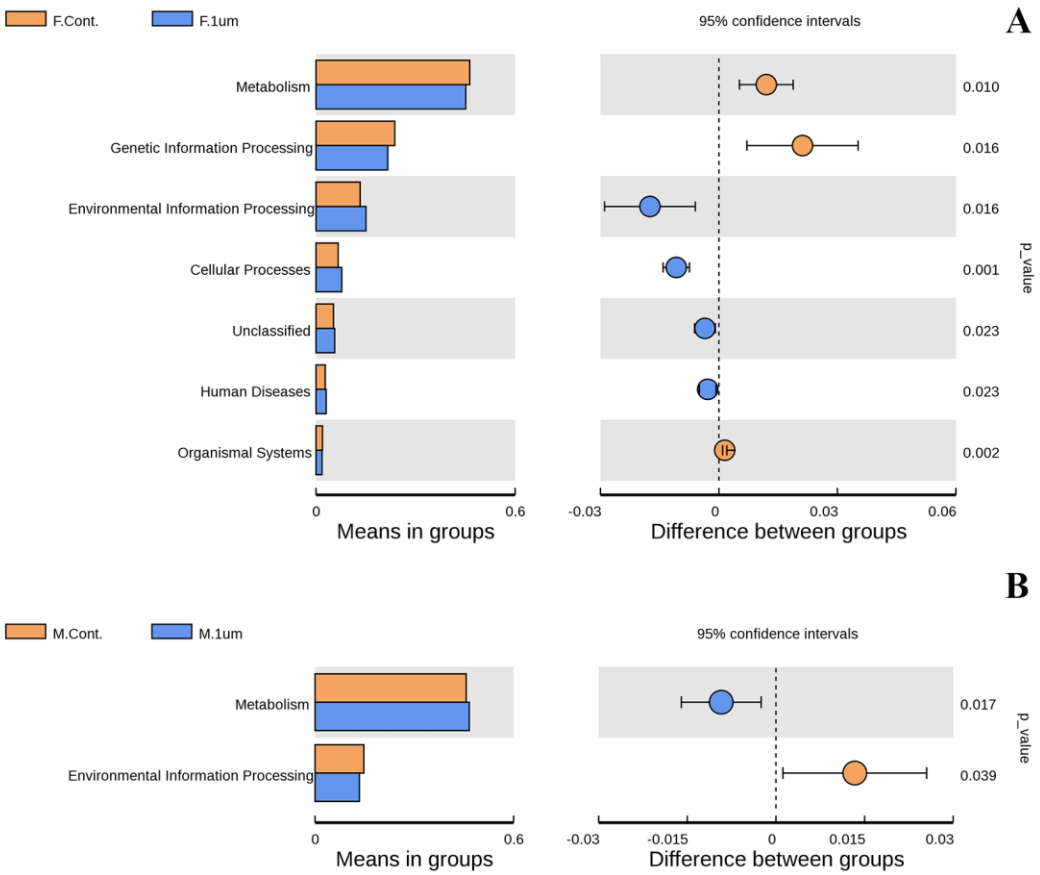


Figure 2.5. Welch's t-test analysis of predicted function (level 1) of zebrafish gut microbiota in female groups by Tax4Fun analysis after exposure to 1μm polystyrene (PS) microplastics for 21 days. A, female groups; B, male groups. F.Cont. represents the female control group, F.1μm represents the female PS microplastic exposed group, M.Cont. represents the male control group, and M.1μm represents the male PS microplastic exposed group.

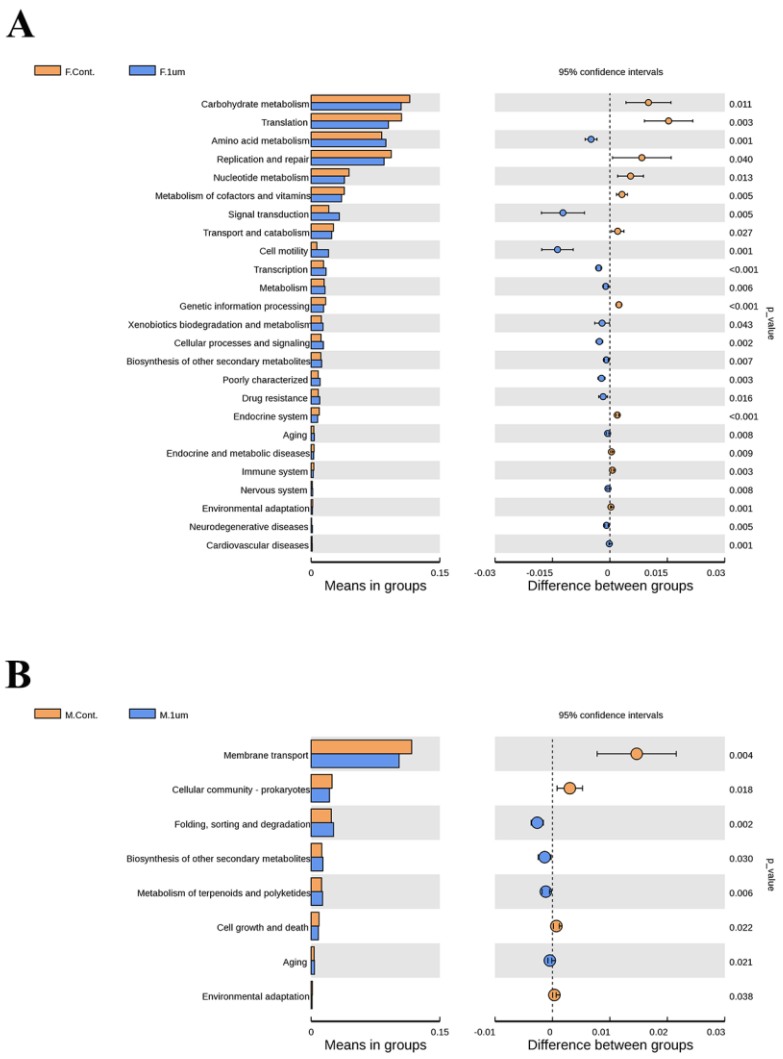


Figure 2.6. Welch's t-test analysis of predicted function (level 2) of zebrafish gut microbiota by Tax4Fun analysis: (A) female groups; (B) male groups. F.Cont. represents the female control group, F.1μm represents the female PS microplastic exposed group, M.Cont. represents the male control group, and M.1μm represents the male PS microplastic exposed group.

Huang et al. (2020) revealed that energy imbalance might lead to reduced replication, recombination and repair pathways in the guppy gut when exposed to PS microplastics. The impact of PS microplastics on energy metabolism, along with reduced energy reserves caused by gut inflammation might lead to under-representation of replication and repair pathways in our study, and an imbalance of energy metabolism can affect growth and reproduction (Watts et al., 2015). It has also been reported that gut microbiota can

regulate sex hormone levels by interactions among their metabolites, the immune system, chronic inflammation, and the gut-brain-gonad axis (He et al., 2021). Some microorganisms are involved in steroid production and oestrogen metabolism (Haque et al., 2022). In our study, the endocrine system pathways (glucagon signalling pathway, PPAR signalling pathway, insulin resistance, oestrogen signalling pathway, adipocytokine signalling pathway, progesterone-mediated oocyte maturation) were under-represented in females, which might be related to sex steroid hormone disorder.

In the male zebrafish group, relatively few functions were influenced by PS microplastics. Enriched pathways included folding, sorting and degradation, and biosynthesis of other secondary metabolites, while under-represented pathways included membrane transport and cellular community-prokaryotes.

2.4.3 Metabolic alterations induced by polystyrene (PS) microplastics

2.4.3.1 Liver metabolomics

In the female PS microplastics treatment, there were a total of 53 metabolites that were significantly different to those in the control. Among them, 8 were up-regulated and 45 were down-regulated (Fig. 2.7 and 2.8A). For the male treatment group, by contrast, fewer metabolites were influenced by PS microplastics; 11 metabolites were up-regulated and 1 metabolite was down-regulated (Fig. 2.7 and 2.8B).

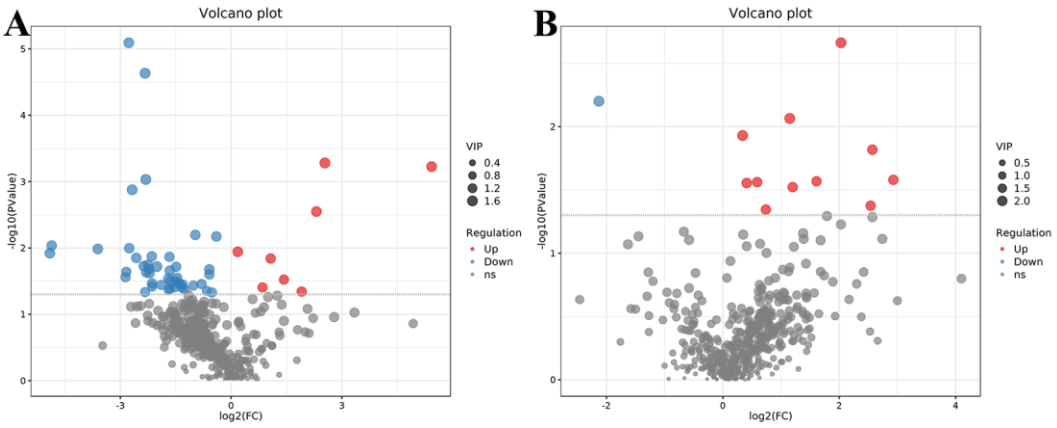


Figure 2.7. Volcano plot of differential metabolites after polystyrene (PS) microplastics exposure: (A) female group; (B) male group.

KEGG metabolic pathway analysis was conducted to better understand the functions of these differential metabolites. As shown in Fig. 2.9, in the female groups of PS microplastics compared to the control, the main metabolic pathways of differential metabolites included the insulin signalling pathway, retinol metabolism, glycolysis/gluconeogenesis, progesterone-mediated oocyte maturation, oocyte meiosis, FoxO signalling pathway, and steroid hormone biosynthesis. In the male groups, the main metabolic pathways impacted by PS microplastics included D-arginine and D-ornithine metabolism, valine, leucine and isoleucine biosynthesis, glycine, serine and threonine metabolism, and steroid hormone biosynthesis.

Small molecule metabolites, including glucose, lipids, amino acids, peptides, and nucleic acids, are crucial indicators that reflect the physiological state and alterations in biosystems (Lin et al., 2023). In this study, the metabolites belonging to carbohydrates (D-glucose, alpha-D-glucose and beta-D-fructose-6-phosphate) in the female PS microplastics treatment showed significant alteration. The altered metabolites were involved in the glycolysis/gluconeogenesis pathway, which is the primary pathway related to energy metabolism (Wang et al., 2022a). Meanwhile, according to KEGG pathway analysis, D-glucose is also involved in the FoxO signalling pathway and insulin signalling pathway, the latter of which is associated with glycolipid metabolism and adipogenesis (Guo et al., 2021). Several studies have found similar results to ours indicating that microplastics could affect glucose metabolism, glycolysis, lipid metabolism, and amino acid metabolism in fish (Ye et al., 2021; Zhao et al., 2021b; Zhu et al., 2024).

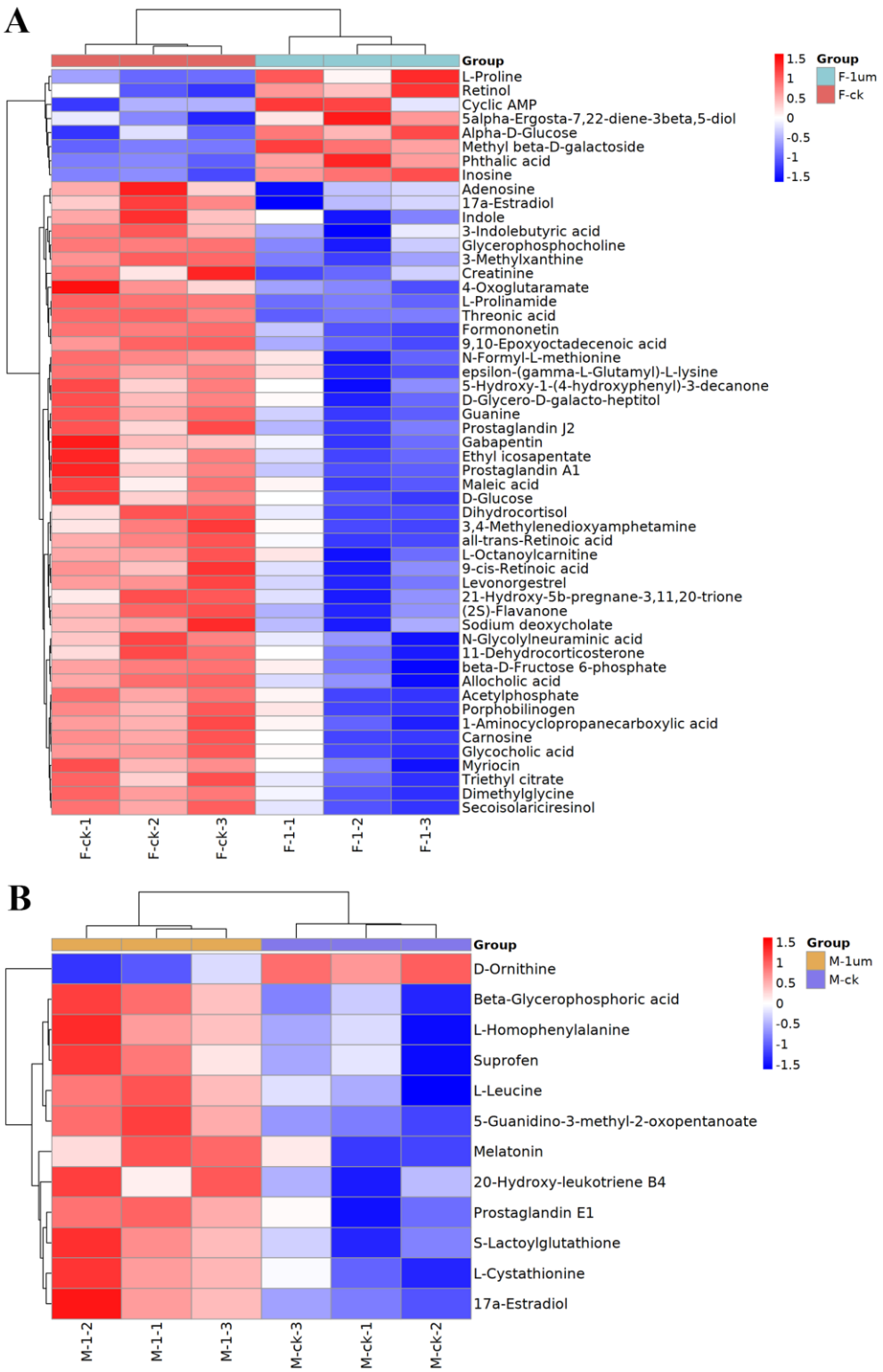


Figure 2.8. Heatmap of the differential metabolites in zebrafish liver after exposure to 1μm polystyrene (PS) microplastics for 21 days. A, female groups; B male groups. F-ck represents the female control group, F-1μm represents the female PS microplastic exposed group, M-ck represents the male control group, and M-1μm represents the male PS microplastic exposed group.

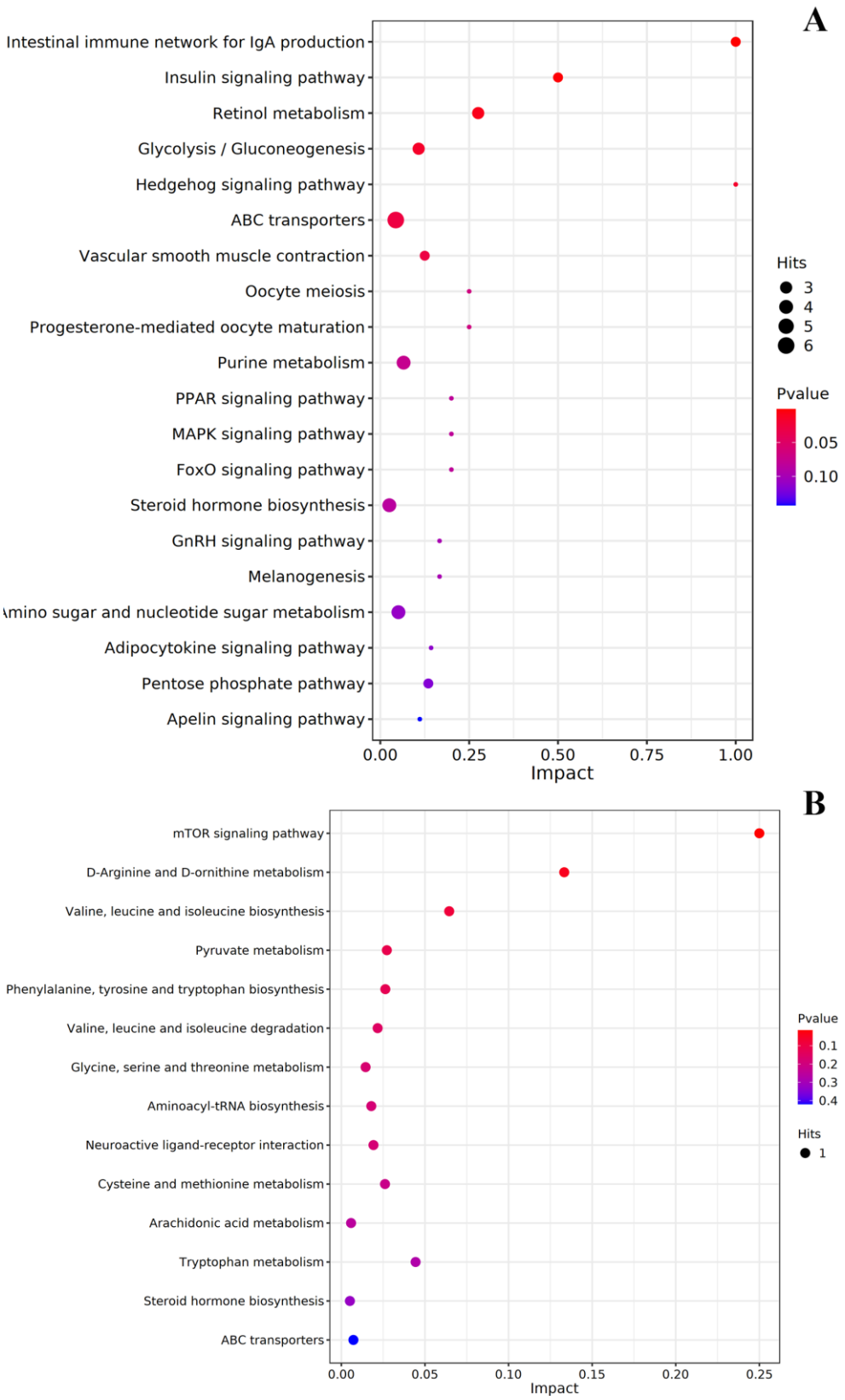


Figure 2.9. KEGG pathway enrichment ($p < 0.05$) in zebrafish liver after exposure to 1µm polystyrene (PS) microplastics for 21 days. A, female groups; B male groups.

Alterations in amounts of several lipid metabolites, such as prenol lipids (retinol, all-

trans-retinoic acid, and 9-cis-retinoic acid), steroids (17a-oestradiol, dihydrocortisol, 21-hydroxy-5b-pregnane-3,11,20-trione, and 11-dehydrocorticosterone), and fatty acyls (9,10-epoxyoctadecenoic acid), were also observed in the female PS microplastics treatment compared to the control. These metabolites are involved in lipid-related metabolic pathways, such as retinol metabolism and steroid hormone biosynthesis, suggesting the disordering of lipid metabolism. The affected lipid and retinol metabolic pathways could interfere with the energy supply for ovarian steroidogenesis, thereby affecting the growth and maturation of zebrafish oocytes (Fang et al., 2022). Meanwhile, KEGG pathway analysis revealed that steroid differences between the PS microplastics treatments and the control involved those associated with hormone biosynthesis, and several pathways related to regulation of reproduction, including progesterone-mediated oocyte maturation and oocyte meiosis. Medriano and Bae (2022) found that polyester fibre could alter the regulation of pivotal sex hormones such as oestrogen and androgen, which might be regulated by gut microbiota. In our study, the endocrine system pathway of gut microbiota was under-represented and pathways of liver metabolism related to reproduction were influenced in females, suggesting the connection between gut microbiota and liver metabolism leads to a decreased E2 level and decreased egg production in female fish.

For male zebrafish treatments, the metabolites affected by PS microplastics, compared to those in the control, were mainly amino acids (such as L-leucine, D-ornithine, and L-cystathionine), suggesting PS microplastics had significant effects on amino acid metabolism, which is linked to basic metabolism, such as lipid and carbohydrate metabolism (Bai et al., 2020; Rui, 2014). Rehman et al. (2024) also found that PS nanoplastics influenced amino acid metabolism of male zebrafish, indicating they affected the energy metabolism pathway.

2.4.3.2 Stable isotope analysis

Stable isotope analysis was used as an integrated whole-organism indicator of nutrient assimilation and metabolic status after PS microplastic exposure. Unlike individual biochemical biomarkers, $\delta^{13}C$ and $\delta^{15}N$ provide cumulative information on

carbon and nitrogen turnover, which may reflect changes in energy metabolism, nutrient absorption, amino acid metabolism and protein balance. Both female and male fish showed a significant decrease of $\delta^{13}C$ and $\delta^{15}N$ following PS microplastic exposure (Fig. 2.10A and B). Ouyang et al. (2021) also found that PS microplastics reduced $\delta^{13}C$ of koi carp, which resulted from the alteration of nutrient absorption. $\delta^{13}C$ has been found to be related to energy metabolic or nutrient stress while $\delta^{15}N$ could be influenced by metabolic changes involving amino acids metabolism, protein synthesis and carbon turnover (Ek et al., 2015). A few studies have shown an interrelationship between gut microbiota and metabolism in zebrafish after exposure to pollutants (Gu et al., 2022; Jiang et al., 2020; Qiao et al., 2019b). The change in gut microbiota after PS microplastic exposure might be related to altered metabolites in liver (mainly carbohydrates, amino acids, nucleic acid), which could contribute to the changes of $\delta^{13}C$ and $\delta^{15}N$.

2.4.4 Sex hormone level and egg production

As shown in Fig. 2.10C-E, for female zebrafish, the concentration of E2 and the E2/T ratio was significantly lower in the treatments compared to the controls, despite no obvious difference in T concentration. Male zebrafish exhibited a significant decrease in T concentration under exposure to PS microplastics and an increased E2/T ratio. Egg production was lower in♀1μm×♂ck treatments and ♀1μm×♂1μm treatments, whereas a slight but not significant decrease was found in ♀ck×♂1μm (Fig. 2.10F).

E2 and T, as gonadal steroids, can regulate the sexual functions of fish, including gametogenesis, gamete maturation and reproduction (Ji et al., 2013). Decreased E2 levels in females could contribute to the reduction of vitellogenesis in fish ovaries (Qian et al., 2020) and inhibit VTG expression, which would have an adverse effect on reproductive processes, leading to decreased egg production (Qian et al., 2020; Reading & Sullivan, 2011), which could be one of the reasons for the decreased egg production in females in our study. Similarly, Gupta et al. (2023) demonstrated that PS microplastics decreased plasma E2 level in female zebrafish, contributing to delayed ovarian development. We found that PS microplastics hindered reproduction of female zebrafish by causing oxidative stress, apoptosis, and hormone level imbalance. Teng et al. (2022) found that

exposure of male and female zebrafish to charged PS microplastics disrupted the reproductive system, sex hormone balance and steroidogenic pathway. Mature female zebrafish should possess higher food consumption and metabolic costs when compared to males, thus any potential disruption of energy or metabolism is expected to result in more serious response in females than in males (Huang et al., 2020; Kurchaba et al., 2020).

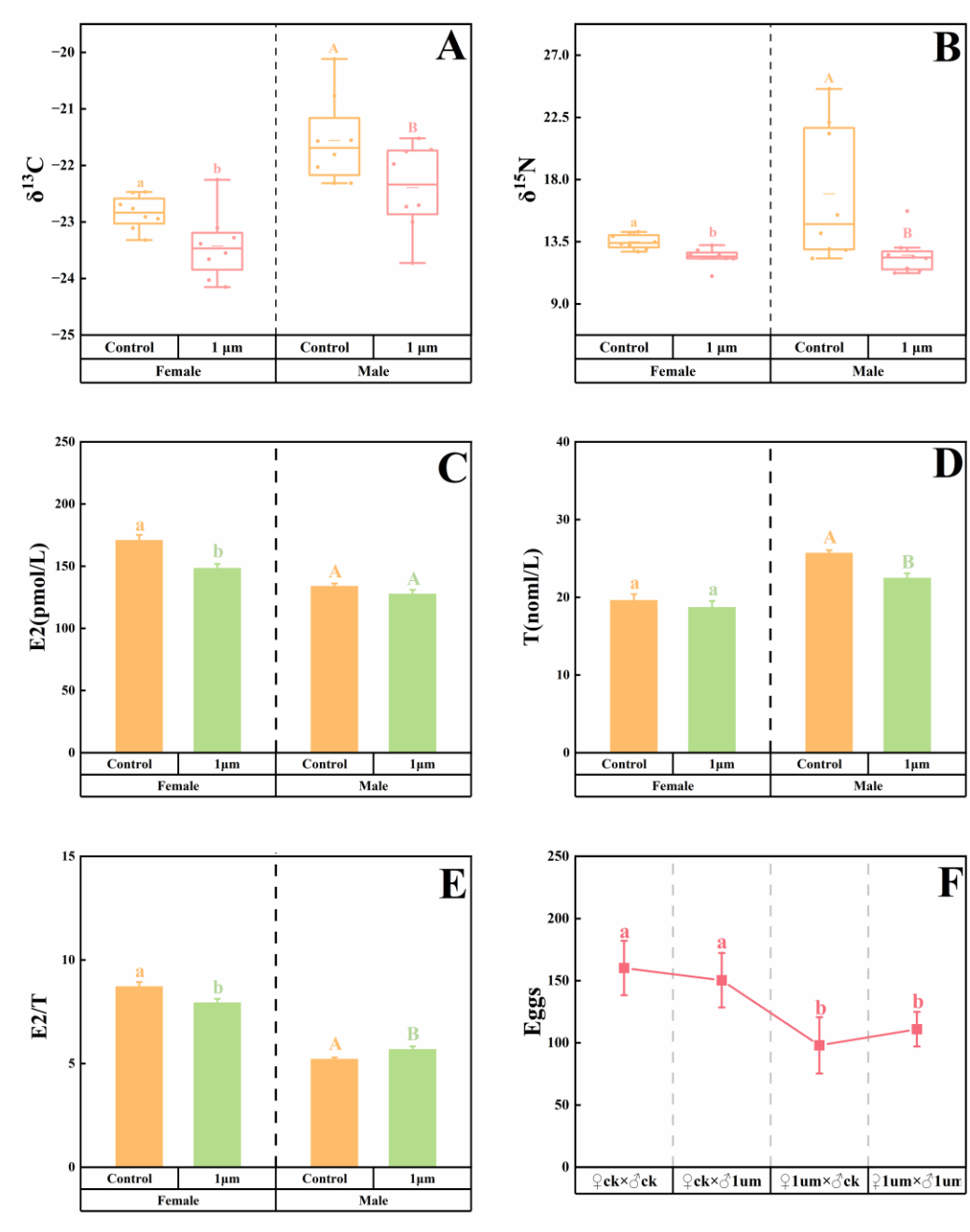


Figure 2.10. Stable isotope analysis, sex hormone level and egg production of zebrafish after exposure to 1μm polystyrene (PS) microplastics for 21 days. Ratios of $\delta^{13}C$ (A) and $\delta^{15}N$ (B) of zebrafish (n=8). Oestradiol (E2) (C), testosterone (T) (D), and E2/T ratio (E)

of zebrafish (n=4). Egg production of paired zebrafish (n=4), ck represents control check group, ♀ represents female fish, and ♂ represents male fish. Data represent mean ± 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 groups.

2.4.5 Conceptual framework for stress-related metabolic and reproductive responses to polystyrene (PS) microplastics in female zebrafish

In zebrafish, various axes govern health regulation, such as the gut-liver axis, hypothalamus-pituitary-gonadal-liver axis (HPGL axis), and gut-brain-gonad axis. The gut-liver axis refers to the bidirectional relationship between the gut, gut microbiota, and liver (Albillos et al., 2020). Previous study found that dysbiosis of gut microbiota increased intestinal permeability, facilitating the translocation of microbial-derived substances to the liver through the gut-liver axis, leading to glycolipid metabolism disorder and liver damage (Liu et al., 2021). Feng et al. (2021) reported that PS microplastics (2 and 200 μm; 3.0 μg/mg) perturbed gut microbiota composition in medaka, along with disrupted glycolipid and energy metabolism, leading to gut-liver axis disruption. In our study, PS microplastic exposure induced gut microbiota dysbiosis together with oxidative stress and altered metabolic pathways in female zebrafish, suggesting a potential link between gut disturbance and hepatic metabolic stress (Fig. 2.11).

The HPGL axis is important in the regulation of the reproductive system, regulating the synthesis of steroid hormones (Teng et al., 2020). Meanwhile, the gut-brain axis can exert influence on the gonads via the endocrine pathway (Wang et al., 2024). The gut microbiota can modulate endocrine control of reproduction by impacting hormone levels, thereby altering their bioavailability and efficacy (Haque et al., 2022). In our study, gut microbiota disturbance was accompanied by underrepresentation of endocrine-related pathways and altered liver metabolic pathways associated with reproduction. These responses were associated with altered sex hormone levels and reduced egg production in female zebrafish.

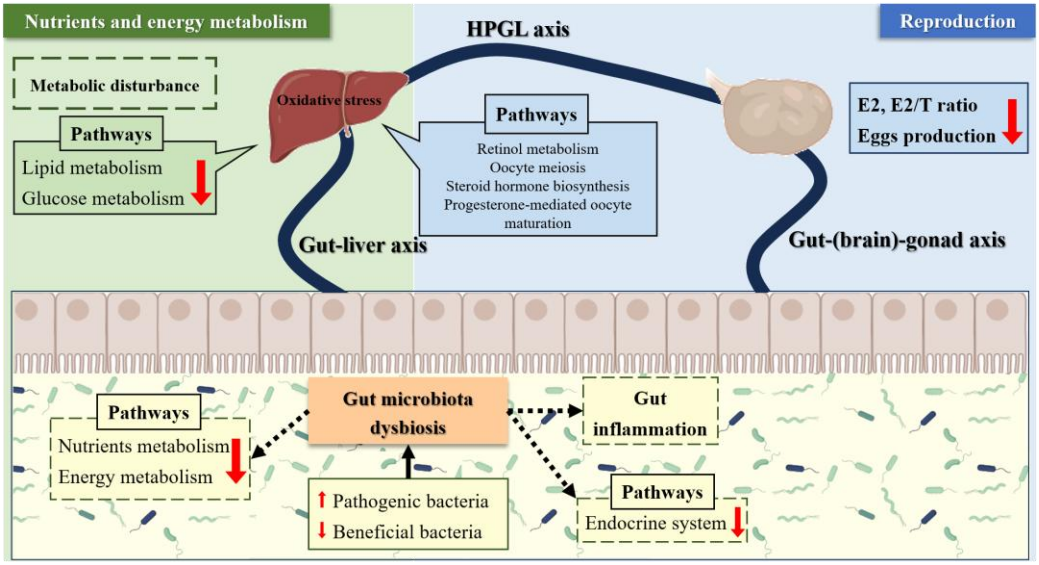


Figure 2.11. Conceptual framework of stress-related biological responses and potential reproductive effects of polystyrene (PS) microplastics in female zebrafish. Solid boxes indicate findings directly supported by the present study, dashed arrows and boxes indicate proposed biological links inferred from the data, and the labelled axes (gut-liver axis, hypothalamus-pituitary-gonadal-liver (HPGL) axis, and gut-brain-gonad axis) represent established conceptual frameworks from previous literature. The figure summarizes stress-related responses, including gut microbiota dysbiosis, oxidative stress, metabolic disturbance and endocrine-related pathway alteration, together with potential adverse reproductive outcomes such as altered sex hormone levels and reduced egg production.

Taken together, the findings of this chapter suggest that PS microplastic exposure in females was associated primarily with stronger stress-related biological responses, including gut microbiota dysbiosis, oxidative stress and metabolic disturbance, together with potential adverse reproductive outcomes such as altered hormone levels and reduced egg production. Importantly, potential impairment of reproductive function may have cascading effects on population dynamics and the overall community structure (Teng et al., 2022; Vannuccini et al., 2016; Wang et al., 2019b). Thus, our results provide evidence that PS microplastics can induce gender-specific biological responses in freshwater fish. Further research should pay increased attention to the gender-specific responses to microplastics in organisms and communities in freshwater environment.

2.4.6 Limitations

Several limitations should be acknowledged with our study. First, in order to investigate the stress-related mechanism of PS microplastics on zebrafish health, the concentration of PS microplastics in our study is higher than typical environmental concentrations in freshwater (though not higher than for total microplastic concentrations (Li et al., 2020)). We used high concentrations specifically to provide us with insights into mechanistic responses. Therefore, the findings provide hazard-based mechanistic evidence under controlled conditions rather than direct prediction of field-level risk. Second, only one polymer type and one particle size were used. 1 μm spherical beads represent a simplified and relatively extreme particle type. Environmental PS microplastics in freshwater systems include a wider range of sizes, shapes and weathering states. Therefore, the findings from this chapter should not be interpreted as representative of all PS microplastics, and no size-dependent toxicity conclusion can be drawn from this single-size design. Third, females and males were housed separately during exposure to enable sex-specific biological assessment, but this design may have altered reproductive physiology because zebrafish reproductive output is influenced by social and mating cues. Therefore, egg production should be interpreted as post-exposure reproductive output under controlled pairing conditions. Fourth, biological sampling was conducted only at the end of the 21-day exposure, so transient early responses, adaptation and recovery dynamics could not be assessed. Fifth, biomass, growth rate and food intake were not measured, which limits interpretation of energy allocation. Finally, the proposed links among gut microbiota, liver metabolism, hormone disruption and reproduction are based on endpoint associations and literature-supported interpretation rather than direct causal testing.

2.5 Conclusion

In summary, our results showed that the biological responses to PS microplastics was related to the ingestion of microplastics rather than the effects of non-specific skin defence. Female zebrafish were more affected than male zebrafish by PS microplastics. PS microplastics significantly influenced zebrafish metabolism, specifically lipid

metabolism, carbohydrate metabolism, energy metabolism and regulation of reproduction. PS microplastics also destroyed the balance of sex hormones and disturbed the reproductive process in zebrafish, especially in females. The adverse impacts on reproduction are related to gut microbiota dysbiosis and metabolic disorder. The overall effects of PS microplastics on zebrafish metabolism and reproduction might influence individual fish health and whole population stability, which would further degrade freshwater ecosystems. The more severe impact of PS microplastics on females, compared to males, may lead to severe effects on zebrafish populations.

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Chapter 3. UV aging enhances stress-related responses to polystyrene nanoplastics in adult zebrafish, especially females

3.1 Abstract

The ecological risks of micro- and nanoplastics are increasingly recognized. However, the extent to which ultraviolet (UV) aging modifies the biological effects of nanoplastics in fish remains insufficiently understood. In this study, adult zebrafish were exposed to 500 nm pristine and UV-aged polystyrene (PS) nanoplastics at 2.5 mg/L for 21 days and health related indicators were analysed. UV photodegradation significantly altered the physicochemical properties of PS nanoplastics, including roughening of surfaces and generation of new oxygen-containing functional groups. Compared with pristine PS nanoplastics, UV-aged PS nanoplastics induced stronger stress-related responses, as indicated by integrated biomarker response analysis. Specifically, UV-aged PS nanoplastics induced oxidative stress and influenced humoral indicators (immunoglobulin M, lipopolysaccharide and triglyceride). Multi-omics analysis revealed that both pristine and UV-aged PS nanoplastics induced gut microbiota dysbiosis and hepatic metabolic disorder, while the female UV-aged PS nanoplastics treatment group was the most affected, showing enhanced biological responses to UV-aged PS nanoplastics and gender-specific impact. The main biological responses associated with UV-aged PS nanoplastics exposure in female zebrafish were the disruption to glycolipid and amino acid metabolism and oxidative stress. Correlation analysis revealed the intimate interplay among factors comprising the gut-liver axis. Our results highlight the enhanced stress-related biological effects of UV-aged PS nanoplastics on freshwater organisms and emphasize the importance of considering the aging process in toxicological and risk assessments of micro- and nanoplastics in freshwater systems.

3.2 Introduction

Micro- and nanoplastic pollution in freshwater ecosystems is attracting increasing

concern. They can be ingested by various freshwater organisms directly, including fish, cladocerans and mussels, leading to adverse impacts (Ding et al., 2021; Grgić et al., 2023). Compared with larger microplastics, nanoplastics may pose additional concerns because their smaller size, larger specific surface area and higher colloidal mobility can enhance interactions with biological surfaces and increase the potential for tissue translocation and cellular-level responses (Gigault et al., 2018; Habumugisha et al., 2023).

Micro- and nanoplastics can affect freshwater organism health at the individual, cellular, and molecular levels (Wang et al., 2022). For example, the accumulation of micro- and nanoplastics has been observed to block the digestive system, hindering nutrient absorption and damaging organism health (Ma et al., 2019; Habumugisha et al., 2023). Ingested micro- and nanoplastics could induce oxidative stress, inflammation, DNA damage and immune inhibition (Cui et al., 2023; Guimarães et al., 2021; Liu et al., 2019a; Qiao et al., 2019a). Small particle size of nanoplastics may further promote close interaction with epithelial barriers, tissue distribution and cellular stress responses (Brun et al., 2019; Teng et al., 2022; Habumugisha et al., 2023). Furthermore, ingested particles may also move through food webs, raising wider concerns about ecological transfer and potential human exposure (Ekvall et al., 2024; Zhang et al., 2020). A comprehensive assessment of the biological effects and risks of micro- and nanoplastics in freshwater ecosystems is therefore necessary, particularly considering that particle properties and behaviour can change during environmental exposure.

After entering freshwater systems, micro- and nanoplastics undergo both abiotic and biological aging processes. Abiotic aging includes ultraviolet irradiation, thermal oxidation, mechanical abrasion and chemical oxidation, which can modify particle morphology, surface charge, hydrophilicity and surface functional groups (Liu et al., 2021). Photoaging is an important process in the aging of micro-and nanoplastics. Ultraviolet (UV)-driven photoaging can induce chain scission, surface roughening, fragmentation and the formation of oxygen-containing functional groups such as carbonyl groups (Dai et al., 2023). Biological aging mainly involves microbial colonisation and biofilm formation on plastic surfaces, resulting in the development of the plastisphere (Esmaeili Nasrabadi et al., 2026). Micro- and nanoplastics can provide a distinct habitat

for microbial communities, and attached biofilms can modify particle density, aggregation, behaviour, surface chemistry and interactions with dissolved organic matter, pollutants and organisms (Amaral-Zettler et al., 2020; McCormick et al., 2014; Miao et al., 2021). These changes may further influence particle bioavailability and biological effects (Rummel et al., 2017; He et al., 2021).

The biological effects of aged micro- and nanoplastics are not consistent across studies and depend on polymer type, particle size, aging process, exposure concentration, exposure duration and test organism. For example, UV-aged polyvinyl chloride (PVC) microplastics increased growth inhibition in *Chlamydomonas reinhardtii*, which was attributed partly to carbonyl group formation and changes in zeta potential (Wang et al., 2020a). Similarly, aged polyethylene (PE) microplastics caused stronger oxidative-stress and cellular responses than virgin particles in the freshwater fish *Perca fluviatilis* (Michailidou et al., 2024). Conversely, other studies have shown that aging does not necessarily increase toxicity, as aged PE microplastics did not enhance acute toxicity to *Daphnia magna* and *Danio rerio*, and aging of polystyrene (PS) nanoplastics did not necessarily enhance toxicity to *Microcystis aeruginosa* (Jemec Kokalj et al., 2019; Wu et al., 2023). Therefore, it remains uncertain about the impacts of aging processes on the biological effects of micro- and nanoplastics, and further study is needed.

Although both abiotic and biological aging are environmentally relevant, this chapter focuses specifically on UV-induced abiotic aging of PS nanoplastics. UV irradiation was selected because it provides a controlled and reproducible way to generate photooxidative changes in PS particles. This approach allowed the study to isolate the effect of abiotic surface oxidation and to compare pristine and UV-aged PS nanoplastics under standardised laboratory conditions. Biofilm-mediated aging was not included in this chapter because it would introduce additional biological variability, including differences in microbial community composition, extracellular polymeric substances and biofilm biomass. These factors could make it difficult to separate the effects of particle surface oxidation from biofilm-associated microbial effects.

After exposure to micro- and nanoplastics, ingestion is the major uptake pathway, however, they can also adhere to fish skin and transfer to other parts of the body

(Kalčíková, 2023; Kim et al., 2021). As an ideal model for toxicology and risk assessment, existing research has shown that micro- and nanoplastics 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 (Brun et al., 2019; Lei et al., 2023; Limonta et al., 2019; Liu et al., 2022). Results from Chapter 2 also demonstrated gender-specific stress-related biological responses and potential reproductive changes in zebrafish exposed to PS microplastics. Furthermore, there has been some research on the impacts of aged microplastics on zebrafish larvae, indicating aggravated neurotoxicity, gut damage, lipid malabsorption, immune dysfunction and growth inhibition (Li et al., 2024; Long et al., 2025; Zhang et al., 2022a). However, the effects of aged PS nanoplastics on adult zebrafish remain less well understood, particularly with respect to sex-dependent gut-liver-associated stress responses.

Accordingly, this study aims to examine whether UV aging modifies stress-related responses to 500 nm PS nanoplastics in adult zebrafish over a 21-day exposure period. The first hypothesis was that UV aging would alter PS nanoplastic surface properties and thereby modify particle-biological interactions. This was assessed through particle characterisation. The second hypothesis was that UV-aged PS nanoplastics would induce stronger stress-related responses than pristine PS nanoplastics, particularly through oxidative stress, immune-related alteration, gut microbiota disturbance and gut-liver metabolic responses. This was tested using biochemical biomarkers, immune-related indicators, gut microbiota analysis, gut and liver metabolomics, correlation analysis and integrated biomarker response version 2 (IBRv2).

3.3 Materials and methods

The methodological design of this chapter was structured to evaluate whether UV aging modified the physicochemical properties and stress-related biological effects of PS nanoplastics in adult zebrafish. First, pristine PS nanoplastics were synthesised, subjected to controlled UVC aging and characterised to determine whether UV exposure altered particle morphology and property. Second, adult female and male zebrafish were exposed separately to pristine or UV-aged PS nanoplastics to compare treatment-related responses

within each sex. Third, immune-related indicators, serum biochemical indicators, hepatic oxidative-stress biomarkers, gut microbiota, gut metabolomics, liver metabolomics, correlation analysis and IBRv2 were used to evaluate gut and liver associated stress responses.

3.3.1 Polystyrene (PS) nanoplastics preparation, aging treatment and characterization

Pristine PS nanoplastics (500 nm) were synthetized in the lab according to methods used in previous studies (Lee et al., 2007; Jiang et al., 2012; Xu et al., 2021b) with modifications. The reaction apparatus is shown in Fig. 3.1. Briefly, ultrapure water was added to a three-neck round-bottom flask equipped with a mechanical stirrer, a condenser and a nitrogen inlet tube. Nitrogen gas was introduced for 30 min to remove dissolved oxygen from the water. Styrene was then added dropwise to the water under continuous stirring at 180 rpm. After stirring at room temperature for 30 min to allow dispersion, potassium persulfate ($K_2S_2O_8$) solution was added dropwise as the initiator. After the addition was completed, the reaction system was heated to 78 °C and maintained for 24 h. Nitrogen gas was continuously supplied throughout the reaction to prevent oxidation of the product. After the reaction, the product was cooled and transferred into 50 mL centrifuge tubes. The suspension was centrifuged at 13,000 rpm for 30 min to separate the solid product from the aqueous supernatant. The supernatant was discarded, and ultrapure water was added to the solid product, followed by ultrasonication for 30 min and centrifugation at 13,000 rpm for 30 min. This washing process with ultrapure water was repeated five times. Subsequently, absolute ethanol was added, followed by ultrasonication for 30 min and centrifugation at 13,000 rpm for 30 min. The ethanol washing process was also repeated five times. The fully washed solid product was then air-dried overnight in a fume hood to allow complete evaporation of residual ethanol, yielding PS nanoplastics. Particle size was controlled by maintaining fixed monomer concentration, initiator concentration, stirring speed, reaction temperature and reaction duration during polymerisation. Particle morphology and dry-state size were examined using transmission electron microscopy (TEM).

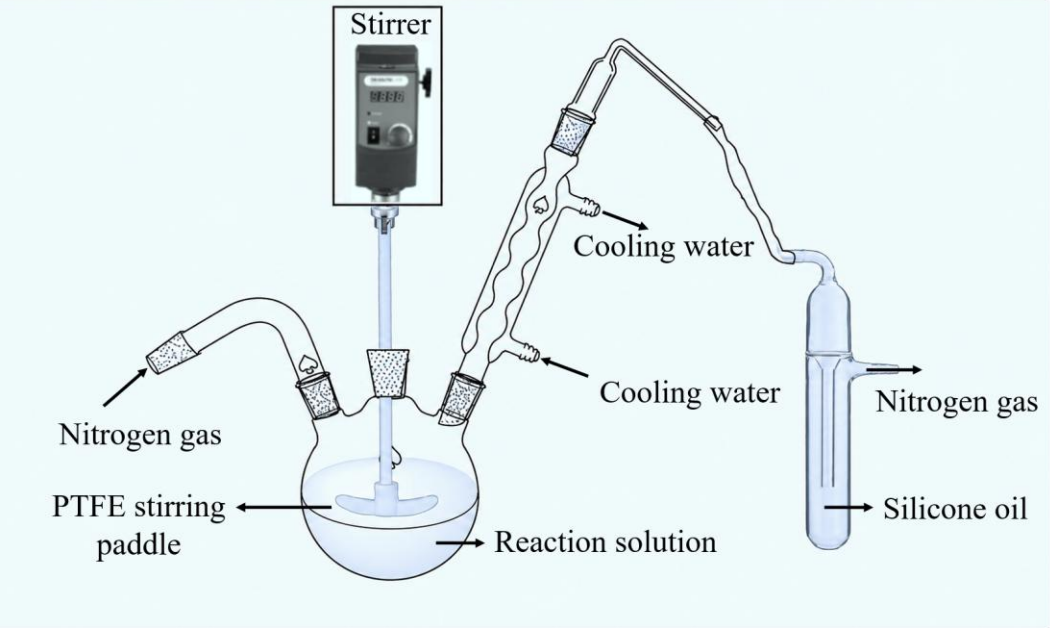


Figure 3.1. Schematic diagram of the reaction apparatus used for the synthesis of polystyrene (PS) nanoplastics.

The pristine PS nanoplastics were placed in a controlled aging chamber and subjected to continuous and uninterrupted UVC irradiation (254 nm; 4 × 20 W; intensity: 75 μW/cm^2) for a total duration of six months. A six-month UV aging duration was selected to generate a clearly measurable degree of photooxidation and surface modification in PS nanoplastics. This duration provided sufficient time for visible colour change, increased surface roughness, increased oxygen content and formation of carbonyl-containing functional groups. Extended UV-aging periods of up to six months have been used in previous studies to characterise photochemical aging of plastic particles, including changes in infrared spectra and carbonyl index under artificial UV or natural sunlight exposure (Zvekic et al., 2022). Six-month weathering experiments have also been applied to expanded polystyrene materials to evaluate progressive surface oxidation and carbonyl index development (Polruang et al., 2025). 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 calculated to quantify the degree of UV-induced oxidation of PS nanoplastics (Liu et al., 2024b). The

band at approximately 1742 cm^{-1} was assigned to carbonyl (C=O) stretching vibrations generated during photooxidation, while the band at approximately 1450 cm^{-1} was assigned to the CH_2/CH bending vibration of the PS backbone and was used as an internal reference band. CI was calculated as the ratio of the integrated peak area of the carbonyl band to the integrated peak area of the reference band:

$$CI = \frac{A_{1742}}{A_{1450}} \quad (3.1)$$

where A_{1742} is the integrated area of the carbonyl absorption band and A_{1450} is the integrated area of the reference band. Before integration, spectra were baseline-corrected and normalised using OMNIC.

3.3.2 Zebrafish culture and experimental setup

Zebrafish (5 months old) were supplied by the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China). After acclimation, zebrafish were randomly assigned to six experimental groups: female control group (F-control), female pristine PS nanoplastics treatment group (FP), female UV-aged PS nanoplastics treatment group (FA), male control group (M-control), male pristine PS nanoplastics treatment group (MP), male UV-aged PS nanoplastics treatment group (MA). Each group contained four replicate glass tanks: 25 zebrafish were included in each tank. The exposure concentration was 2.5 mg/L. Assuming spherical PS nanoplastics with a diameter of 500 nm and a PS density of 1.05 g/cm^3, this mass concentration corresponds to approximately 3.6×10^7 particles/mL. This 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. After 21 days of exposure, zebrafish were anesthetized and sacrificed in ice water before obtaining the tissues.

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

Mucus IgM, serum LPS and TG, and hepatic oxidative-stress biomarkers were

measured to evaluate immune-related, metabolic and oxidative responses after PS nanoplastic exposure. Mucus IgM was selected as an indicator of mucosal immune response. Serum LPS was measured as an indicator associated with gut-derived bacterial endotoxin exposure and possible intestinal barrier disturbance. Serum TG was measured to assess lipid-metabolism-related response. Hepatic ROS, SOD and MDA were measured to evaluate oxidative-stress status in the liver. Skin mucus was collected according to methods outlined in a previous study (Wang et al., 2019) (Text S3.1). 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).

3.3.4 Integrated biomarker responses (IBR) analysis

Integrated biomarker response version 2 (IBRv2) analysis was conducted to summarize measured stress-response indicators into one general index (Sanchez et al., 2013). The stress-related indicators included ROS, SOD, MDA, LPS, TG, and IgM. The calculation formulae are listed in Text S3.2.

3.3.5 Gut microbiome analysis

Gut microbiome analysis was performed to determine whether pristine and UV-aged PS nanoplastics altered gut microbial community composition and predicted microbial functions. The gut samples were collected, frozen in liquid nitrogen and then preserved at -80 ℃. Total genomic DNA was extracted from gut samples using the CTAB/SDS method, and DNA quality and concentration were checked by 1% agarose gel electrophoresis before dilution to 1 ng/μL. The V3-V4 region of the bacterial 16S rRNA gene was amplified using specific primers with barcodes. PCR amplification was performed using Phusion High-Fidelity PCR Master Mix, followed by agarose gel verification, pooling of PCR products in equal proportions and purification using the Universal DNA PCR Purification Kit. Sequencing libraries were generated using the

NEBNext Ultra DNA Library Prep Kit for Illumina. Library quality was assessed using the Agilent 5400 system and quantified by real-time PCR. Sequencing was performed on an Illumina NovaSeq platform to generate 250 bp paired-end reads. Raw reads were merged using FLASH and quality-filtered using QIIME, while chimeric sequences were removed using UCHIME. High-quality sequences were clustered into operational taxonomic units (OTUs) at 97% similarity using UPARSE. Taxonomic annotation was performed against the Silva database using the Mothur algorithm. Alpha and beta diversity were calculated using QIIME, and microbial community structure was visualised using PCoA. Predicted microbial functions were analysed using PICRUSt2, and pathway information was obtained from the KEGG database. Differences in predicted functional abundance among groups were further evaluated using Metastats differential analysis. The sequencing data were deposited in the NCBI database under BioProject accession number PRJNA1265363. The details of DNA extraction, 16s rRNA gene sequencing, and data analysis are listed in Text S3.3.

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

Gut and liver metabolomic analyses were conducted to determine whether pristine and UV-aged PS nanoplastics altered metabolic regulation along the gut-liver axis. The gut and liver samples were collected, frozen in liquid nitrogen and then preserved at -80 ℃ for further analysis. Metabolites were extracted using methanol/acetonitrile (1:1, v/v), followed by sonication at 4 °C and centrifugation. The supernatant was collected, freeze-dried and reconstituted in 30% acetonitrile before UHPLC-ESI-MS/MS analysis. Metabolomic profiling was performed using an UPLC-Orbitrap MS system. Separation was carried out on a Waters HSS T3 column using water and acetonitrile, both containing 0.1% acetic acid, as mobile phases. High-resolution MS data were acquired using a Q Exactive HFX Hybrid Quadrupole Orbitrap mass spectrometer equipped with an electrospray ionisation source. Raw MS data were processed using Progenesis QI for peak detection, alignment and quantification. Multivariate analyses, including PCA and OPLS-DA, were performed in R to visualise metabolic differences among groups. Model reliability was evaluated using cross-validation and permutation testing. Differentially

expressed metabolites were identified based on VIP > 1, fold change > 1.5 or < 0.67, and $p < 0.05$. Identified metabolites were annotated using the KEGG Compound database and mapped to KEGG pathways for pathway enrichment and biological interpretation. The details of sample preparation and extraction, UPLC conditions, LC-MS/MS analysis, data analysis and interpretation and bioinformatics analysis are provided in Text S3.4. 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).

3.3.7 Data analysis

The data are presented as mean ± standard deviation. Normality was assessed using the Shapiro-Wilk test, and homogeneity of variance was assessed using Levene's test. Normally distributed data with homogeneous variance were analysed using one-way ANOVA followed by Tukey's post hoc test. If the data were normally distributed but variance homogeneity was not met, Welch ANOVA followed by Games-Howell post hoc test was used. A significance threshold of $p < 0.05$ was applied. Exact p-values for the statistical comparisons shown in the Chapter 3 figures are provided in Table S3.1. Because female and male zebrafish were housed, exposed and sampled separately, this study did not formally test sex × treatment interactions using a two-way ANOVA. Instead, within-sex comparisons were used to avoid confounding from baseline differences between females and males and to identify treatment-related changes relative to sex-specific controls.

3.4 Results and discussion

3.4.1 Characterization of pristine and UV-aged polystyrene (PS) nanoplastics

Smooth surfaces were observed in pristine PS nanoplastics while UV-aged PS nanoplastics had rough surface and bulges (Fig. 3.2). PS nanoplastics was observed to

change colour from white to yellow after the UV aging process. XPS spectra showed that the O 1s proportion of UV-aged PS nanoplastics increased from 8.03% to 19.92%, while the C 1s proportion of UV-aged PS nanoplastics decreased from 91.97% to 80.08%. FTIR results (Fig. 3.2D) 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 UV-aged PS nanoplastics was 0.43 ± 0.01 and 3.08 ± 0.003, respectively.

Several studies have demonstrated that laboratory accelerated oxidation of micro-and nanoplastics could simulate the long-term aging process in the natural environment, and UV photodegradation can accelerate the aging process and alter physicochemical properties of micro- and nanoplastics (Duan et al., 2023; Liu et al., 2019b; Wu et al., 2020). As with our study, Long et al. (2025) also observed surface cracks in aged PS nanoplastics (80 nm); Chen et al. (2021) found that UV radiation could change the colour and increase O 1s proportion of PS microplastics (1.014 ± 0.130 μm), indicating that PS microplastics had been oxidized. The generation of new oxygen-containing functional groups from FTIR analysis further demonstrated this process had occurred. The mechanism may be the formation of peroxy radicals and hydroperoxide intermediates, which subsequently results in the peroxy radicals undergoing chain scission to form carbonyl compounds (Yousif & Haddad, 2013). Combined, our results show that UV photodegradation altered the physicochemical properties of PS nanoplastics.

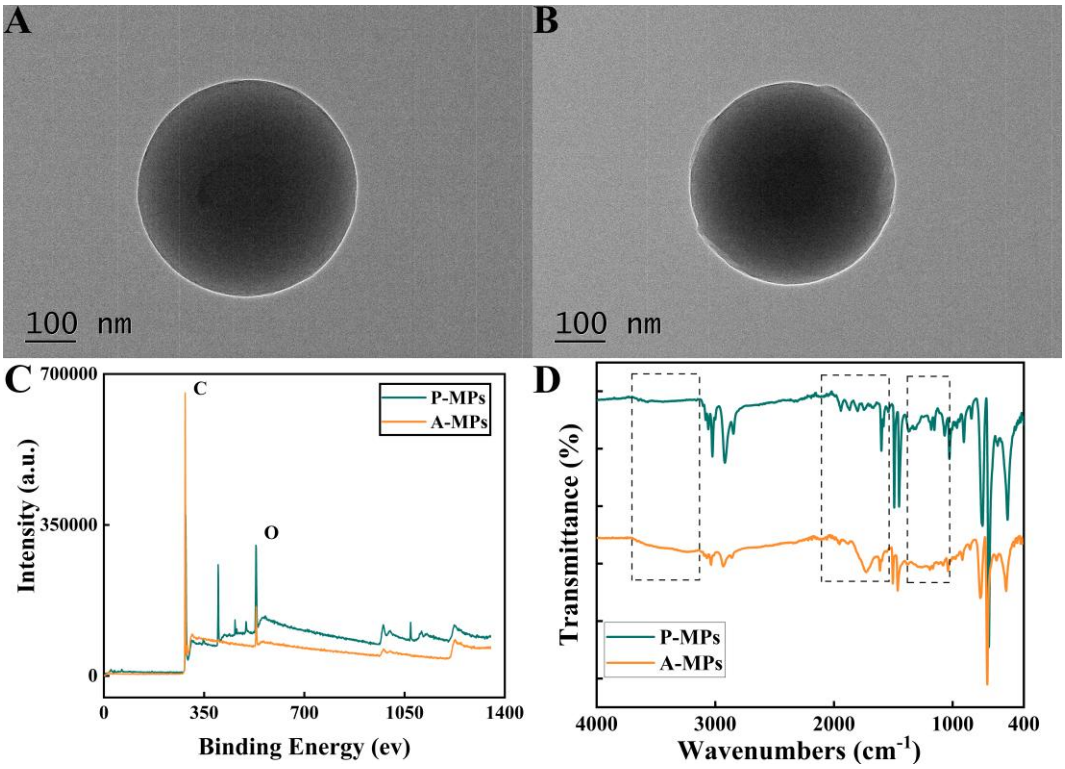


Figure 3.2. Characterization of pristine and UV-aged polystyrene (PS) nanoplastics. Transmission electron microscope (TEM) images of PS nanoplastics after UV aging process. A, pristine PS nanoplastics; B, UV-aged PS nanoplastics. X-ray photoelectron spectroscopy (XPS) (C) and Fourier transform infrared spectroscopy (FTIR) (D) spectra of pristine and UV-aged PS nanoplastics.

3.4.2 Humoral indicators, oxidative stress and integrated biomarker response (IBR) analysis

Humoral indicators were determined in blood serum and skin mucus. As shown in Fig. 3.3A, both pristine and UV-aged PS nanoplastics significantly increased immunoglobulin M (IgM) level in females. The increase of IgM level in the FA group is greater than that in the FP group. The skin mucus of fish is a barrier against various chemical, physical and biological stressors, regarded as the first line of defence against infection (Benhamed et al., 2014). As one of the immune parameters in mucus, IgM serves as an immune effector in the humoral immune system of fish (Salinas et al., 2021). To our knowledge, there is little research focusing on the effects of nanoplastics on zebrafish mucus immunity. Yang et al. (2020) showed that PS microplastics 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 nanoplastic groups, and the FA group showed a greater increase in mucus compared to the FP group, indicating PS nanoplastics might induce immunostimulatory effects on female zebrafish and UV-aged PS nanoplastics induced more of these effects.

Fig. 3.3B and C show that UV-aged PS nanoplastics raised lipopolysaccharide (LPS) and triglyceride (TG) content significantly in both genders. However, no differences were found between controls and pristine PS nanoplastic groups. UV-aged PS nanoplastics 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 nanoplastic treatments (Fig. 3.3D-F).

Gut microbiota can influence host health by producing metabolites such as the intracellular toxin - LPS (Caesar et al., 2010). The release of LPS is related to changes in the composition of gut microbiota (Luo et al., 2021a). Luan et al. (2024) similarly found that polyglycolic acid microplastics 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 gram-negative bacteria and upregulation of LPS biosynthesis could increase LPS level in the gut (section 3.4.3.1), may be associated with gut inflammatory signalling (Qiao et al., 2019b). The increased serum LPS level in UV-aged PS nanoplastic groups suggested possible gut barrier disturbance, leading to the discharge of gut LPS to the bloodstream (Papa et al., 2023), which may further induce immune and inflammatory responses and oxidative stress in the liver (Liu et al., 2024b; Luo et al., 2021b).

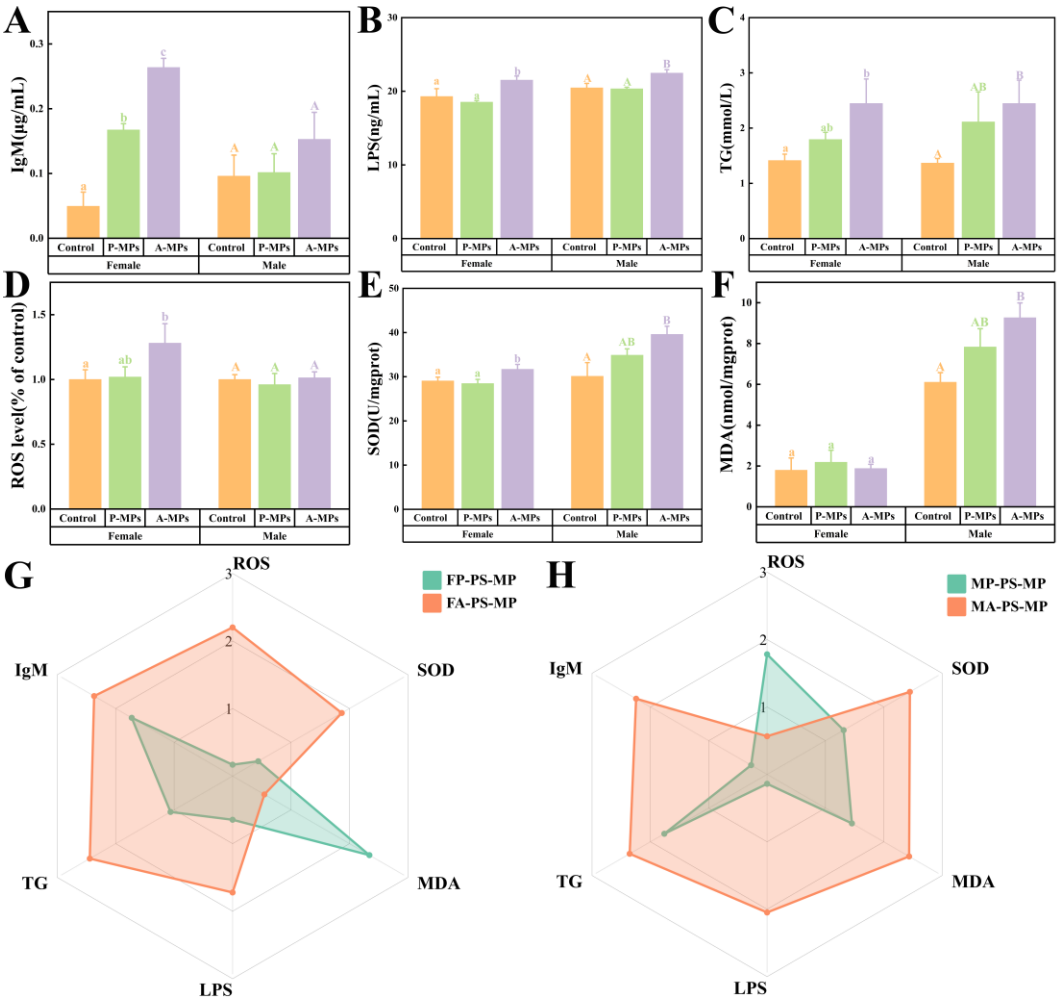


Figure 3.3. Immunoglobulin M (IgM) level (A), lipopolysaccharide (LPS) level (B), triglyceride (TG) level (C), reactive oxygen species (ROS) level (D), superoxide dismutase (SOD) activity (E), malondialdehyde (MDA) content (F), and integrated biomarker response (IBR) analysis (G, female zebrafish; H, male zebrafish) of zebrafish after exposure to pristine and UV-aged polystyrene (PS) nanoplastics for 21 days. Data represent mean ± 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.

Oxidative damage is one of the major stress-related mechanisms of micro-and nanoplastics to zebrafish (Bhagat et al., 2020). In our study, UV-aged PS nanoplastics induced oxidative stress and lipid peroxidation in the zebrafish liver, which may be explained by their changed surface characteristics, including the increase of oxygen-containing functional groups and free radicals (Zhong et al., 2023). The oxidative

imbalance could damage hepatic cells and disrupt the homeostasis of liver lipid metabolism (Emami et al., 2020; Pan et al., 2018). As a widely present lipid compound in organisms and one of the most important forms of energy storage, the primary site of TG synthesis is the liver (Wang et al., 2020b). The increase in serum TG levels indicated an increase in liver lipid levels and their release into the systemic circulation. The accumulation of TG in the liver will induce liver dysfunction and damage to liver cells, leading to liver lipid metabolism disorders (Zheng et al., 2021), and therefore the increased content of TG, ROS, SOD and MDA observed in our study in response to UV-aged nanoplastic exposure suggests lipid metabolism dysbiosis and oxidative damage occurred in the liver.

The IBR analysis integrated multi-indicator responses (Fig. 3.3 G and H). The value of the IBRv2 index in the UV-aged PS nanoplastic treatment group was higher than that in the pristine PS nanoplastic treatment in both genders. A high IBR value usually indicates an increase in stress responses in the test organism (Teng et al., 2021). The higher IBRv2 value indicated a stronger integrated stress-response pattern in the UV-aged PS nanoplastic treatment groups.

3.4.3 The stress-related biological responses on zebrafish induced by pristine and UV-aged polystyrene (PS) nanoplastics through the gut-liver axis

By inducing oxidative stress and the disruption of humoral indicators in zebrafish, UV-aged PS nanoplastics induced stronger stress-related responses than pristine PS nanoplastics. To clarify how pristine and UV-aged PS nanoplastics affect stress-related biological responses in zebrafish, it is necessary to further analyse molecular processes along gut-liver axis to explain the changes in gut microbiome and metabolism.

3.4.3.1 Gut microbiota composition and predicted function

The Venn diagram (Fig. S3.1 A and B) showed that PS nanoplastics changed the number of OTUs of gut microbiota compared to the control group. Consistent results were found by PLS-DA analysis (Fig. S3.1C and D), with separated clusters observed among

different groups in both genders.

The relative abundance of gut microbiota composition in the control, pristine and UV-aged PS nanoplastic treatment groups was exhibited at the phylum level in Fig. 3.4. Firmicutes and Bacteroidota increased while Proteobacteria and Actinobacteriota decreased after exposure to pristine PS nanoplastics. UV-aged PS nanoplastics increased Proteobacteria and Bacteroidota and reduced Firmicutes and Actinobacteriota. In male zebrafish, pristine and UV-aged PS nanoplastics both increased Firmicutes, Actinobacteriota and Bacteroidota while decreasing Proteobacteria.

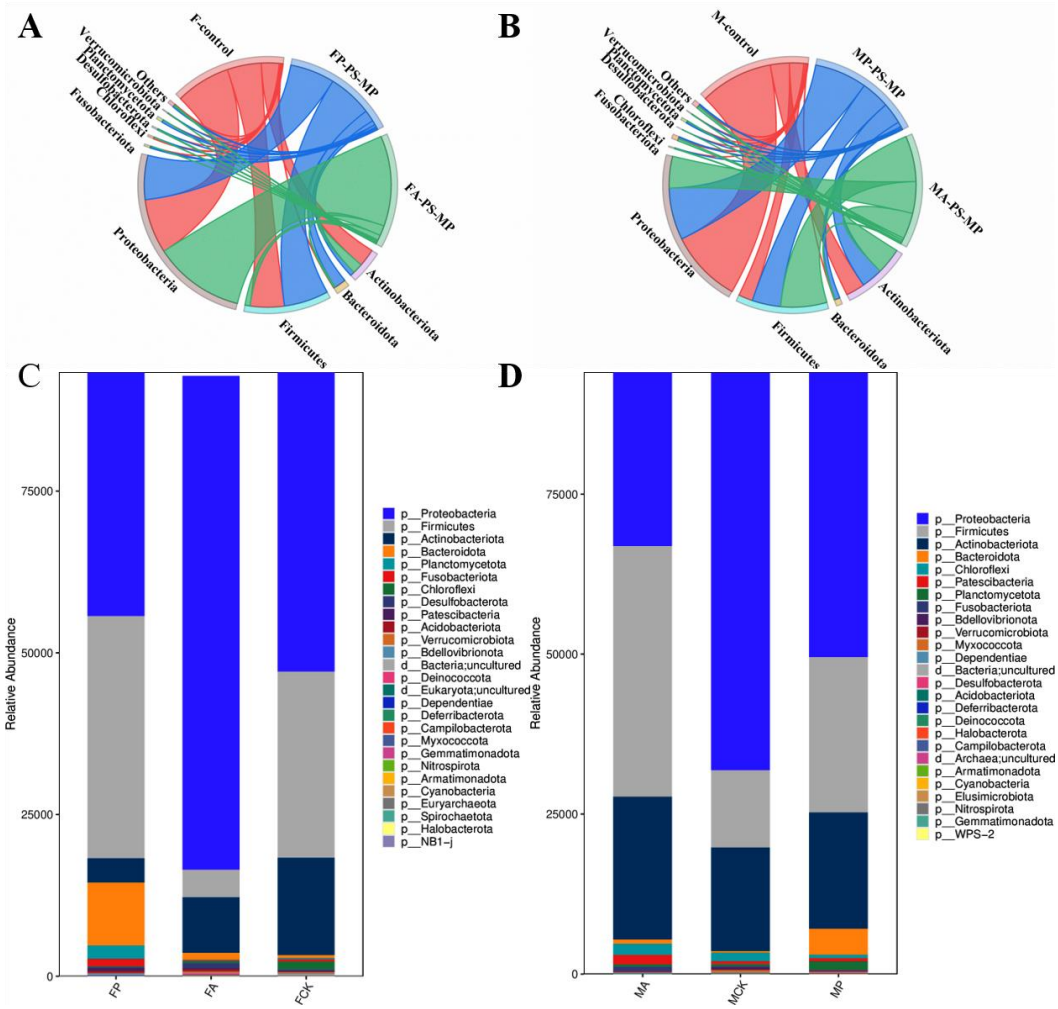


Figure 3.4. Gut microbiota composition at phylum level among different treatment groups after exposure to pristine and UV-aged polystyrene (PS) nanoplastics for 21 days. A and C, female zebrafish; B and D, male zebrafish.

It has been previously demonstrated that Proteobacteria play an important role in gut

inflammation (Qiao et al., 2019b). In our study it is worth noting that UV-aged PS nanoplastics increased the relative abundance of Proteobacteria only in female zebrafish, suggesting potential gender-specific impacts on gut inflammation and oxidative stress might be induced by UV-aged PS nanoplastics. This finding is of concern as inflammation in the gut may also change gut permeability and lead to the release of intracellular toxins (such as LPS) into the bloodstream (Alhamoruni et al., 2012; Papa et al., 2023), which we observed (section 3.4.2). The relative abundance of genus *Pseudomonas* and *Aeromonas* increased in the FA group (Fig. 3.5A). These genera are fish pathogens and can cause several fish diseases, such as lung infections, enteritis, and ulcer disease (Oliver et al., 2000; Yu et al., 2019). Thus, the microbiota changes in the FA group may have affected the health of female zebrafish, potentially induced inflammation and influencing metabolism and liver-related indicators.

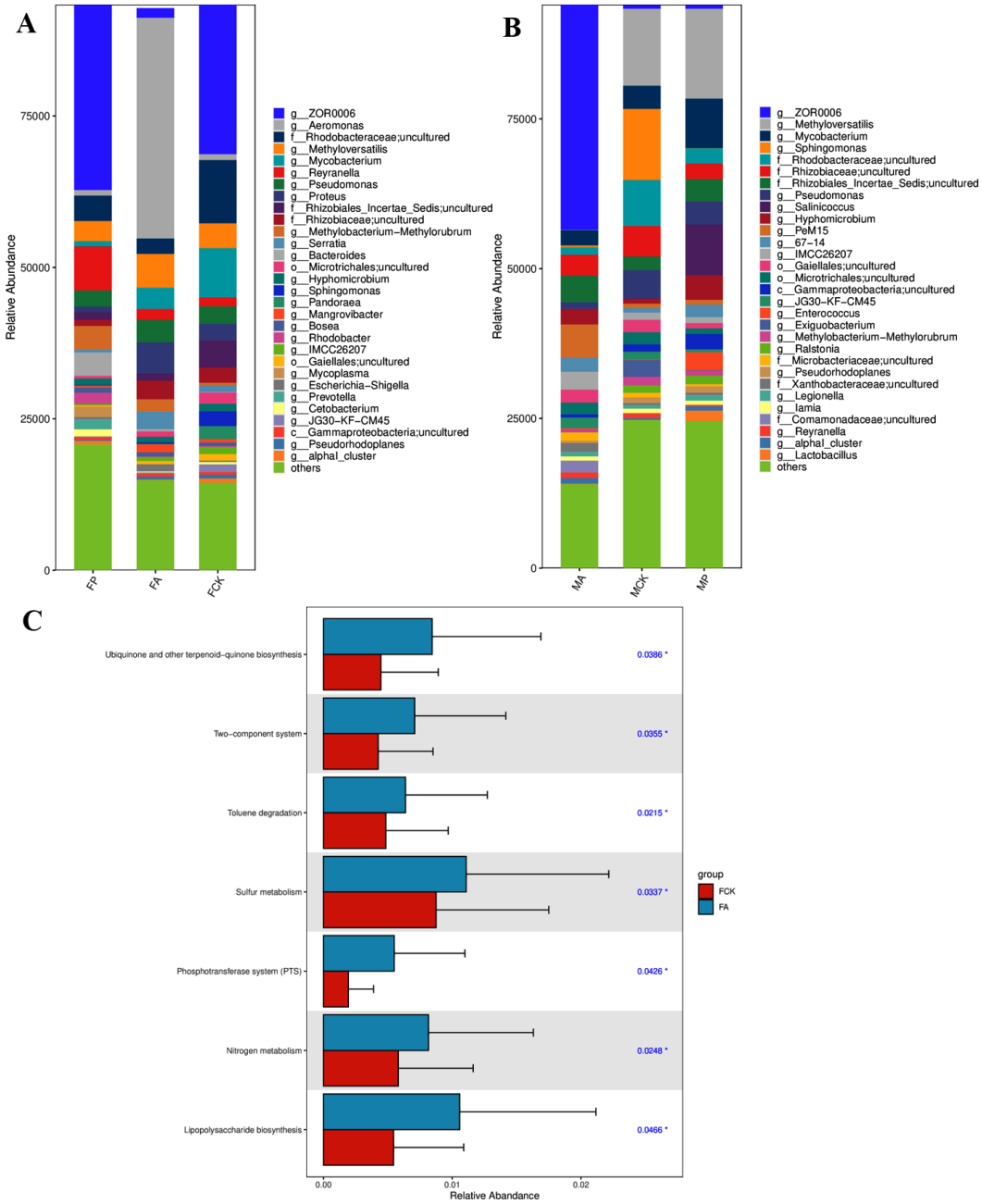


Figure 3.5. The relative abundance of gut microbiota at genus level after pristine and UV-aged polystyrene (PS) nanoplastic exposure for 21 days, A, female groups; B, male groups. C, predicted function of zebrafish gut microbiota between F-control group and FA group by PICRUSt2 analysis.

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 (Dehler et al., 2017; Xu et al., 2021a). In contrast to several studies (Li et al., 2023a; Liu et al., 2023; Zhao et al., 2024), Firmicutes and Bacteroidetes increased and

Proteobacteria reduced in the FP, MP, and MA groups compared to controls. Gu et al. (2020) 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 (Zuo et al., 2018), our results highlight the potential for dysbiosis in the gut microbiota by PS nanoplastics, which may further result in oxidative stress and inflammatory responses.

Due to the FA group having specific increased pathogens and being the most affected in liver metabolism (section 3.4.3.4), predicted functions of gut microbiota in the FA group was assessed 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. 3.5C).

We found that UV-aged PS nanoplastics 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 (Blötz & Stülke, 2017). Several studies also demonstrated the potential modification of microbial element metabolism functions induced by micro- and nanoplastics, including C/N/S/P metabolism (Feng et al., 2021; Li et al., 2023b). 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 (Guo et al., 2013). Thus, the upregulation of LPS biosynthesis may indicate a more invasive gut microbiota.

3.4.3.2 Gut metabolomic analysis and KEGG pathway analysis

As there were some different changes of gut microbiota in the FA group, female zebrafish were selected to further investigate the impacts of pristine and UV-aged PS nanoplastics on gut metabolism. Principal component analysis (PCA) showed that the FA group significantly deviated from the F-control group, while no deviation was found in the FP group (Fig. 3.6A). In the FA group, among the 129 DEMs, 91 were up-regulated

and 38 were down-regulated, while only 37 DEMs (21 were up-regulated and 16 were down-regulated) changed in the FP group (Fig. 3.6B and C). Hierarchical clustering (Fig. S3.2) also demonstrated that more DEMs were influenced in the FA group compared to the FP group. Most DEMs in the FA group belong to lipids, nucleosides, nucleotides, and analogues, and organic acids; while DEMs in the FP group mainly belong to lipids, organic nitrogen compounds, and organic acids.

KEGG pathway analysis (Fig. 3.7A and B) showed that more metabolic pathways were influenced in the FA group compared to the FP group. The enriched pathways in the FA group included lipid metabolism, amino acid metabolism, and nucleotide metabolism, while pristine PS nanoplastics mainly affected lipid metabolism, amino acid metabolism and endocrine system.

Lipid metabolism pathway is significantly influenced by UV-aged PS nanoplastics. As one of the fundamental gut functions, lipid metabolism is important for energy production and metabolism (Qiao et al., 2019b). Similarly, several studies have shown that micro- and nanoplastics can induce disruptions in gut lipid metabolism in fish, causing adverse biological responses (Qiao et al., 2019a; Yao et al., 2024). Rossi et al. (2013) demonstrated that PS nanoplastics could readily permeate into lipid membranes, altering their structure. Bansal et al. (2012) found that PS nanoplastics can bind to apolipoprotein A-I in fish to interfere with lipid metabolism.

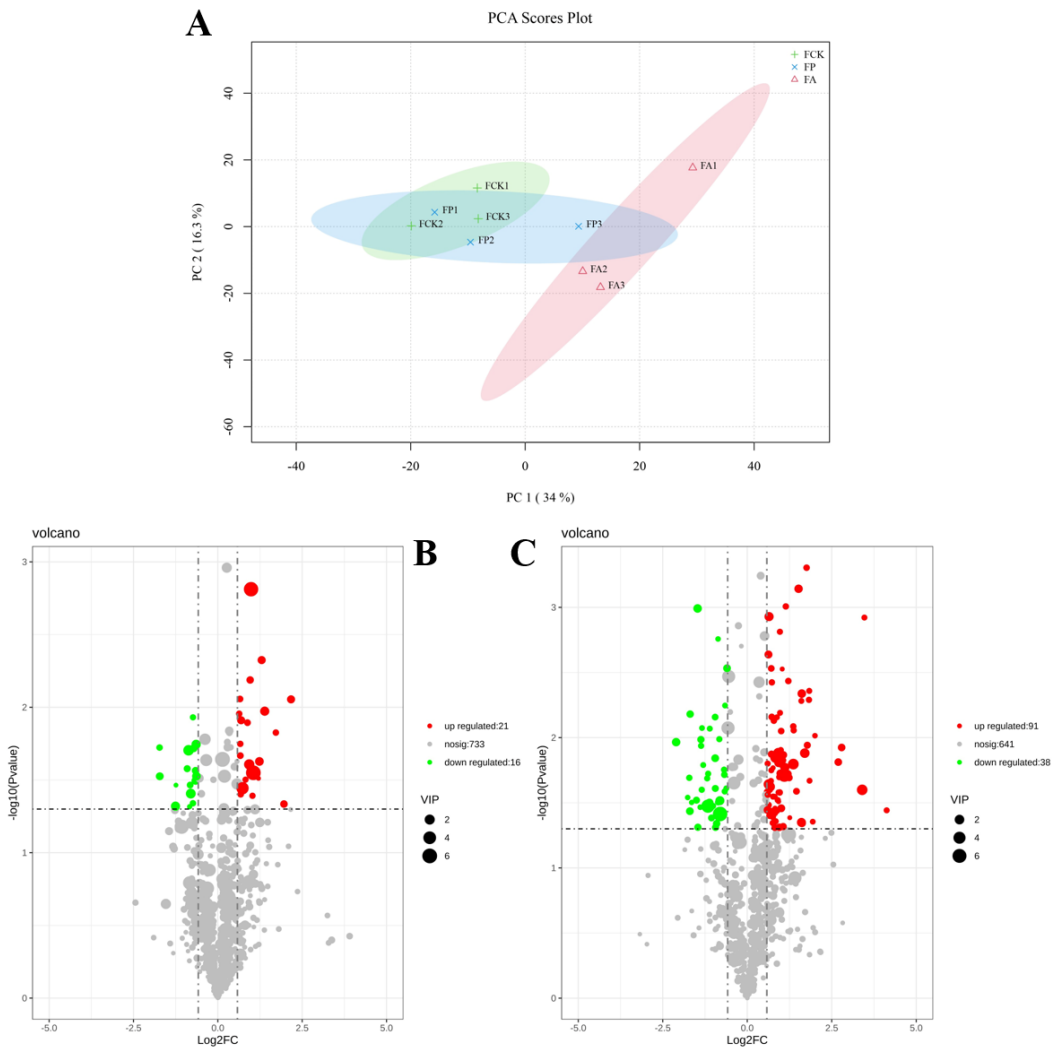


Figure 3.6. Principal components analysis (PCA) of the gut metabolites of the F-control group, FP group and FA groups. Volcano plot of differential metabolites in gut after pristine and UV-aged polystyrene (PS) nanoplastic exposure for 21 days, B, F-control group and FP group; C, F-control group and FA group.

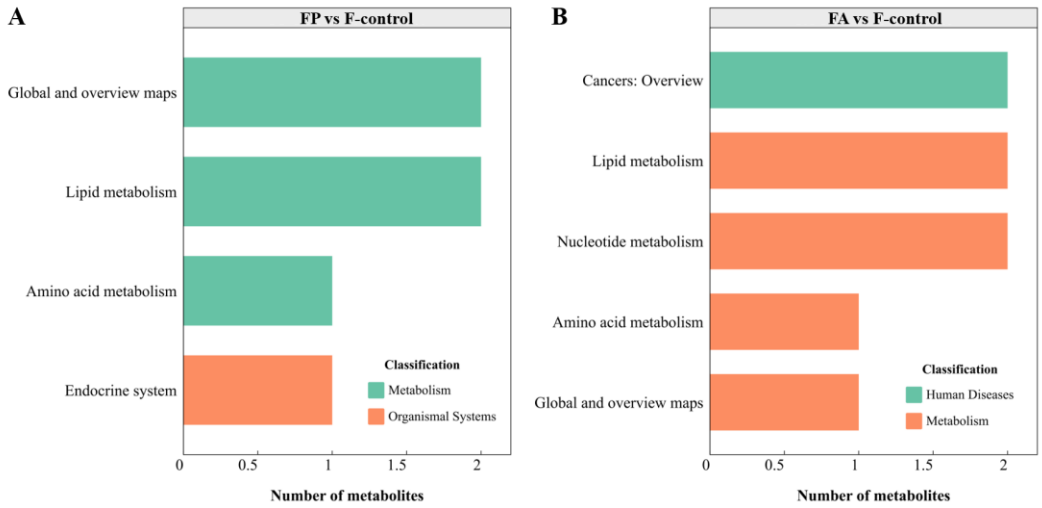


Figure 3.7. KEGG pathway annotation in female zebrafish gut after exposure to pristine and UV-aged polystyrene (PS) nanoplastics for 21 days. A, FP vs F-control group; B, FA vs F-control group.

3.4.3.3 Correlation between influenced gut microbiota and gut differentially expressed metabolites (DEMs) in female zebrafish

Gut microbiota and metabolites are closely linked in the intestinal environment, as microbial communities can influence nutrient processing, lipid metabolism, amino acid metabolism and host stress responses. Therefore, Pearson correlation analysis was performed to explore whether the gut microbial changes observed after PS nanoplastic exposure were associated with alterations in gut metabolic profiles. This analysis was used to identify potential microbiota-metabolite associations and to provide supporting evidence for gut-level stress-related responses. In the FP group, only Actinobacteriota was related to 8-hydroxy-5Z, 9E, 11Z, 14Z-eicosatetraenoic acid and 17-aminogeldanamycin (Fig. 3.8). In the FA group, gut microbiota was closely related to lipid metabolites. Specifically, Proteobacteria was closely related to larsucosterol, delta-24-cholesterol, SM (d16:2(4E, 8Z)/PGJ2), and 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.

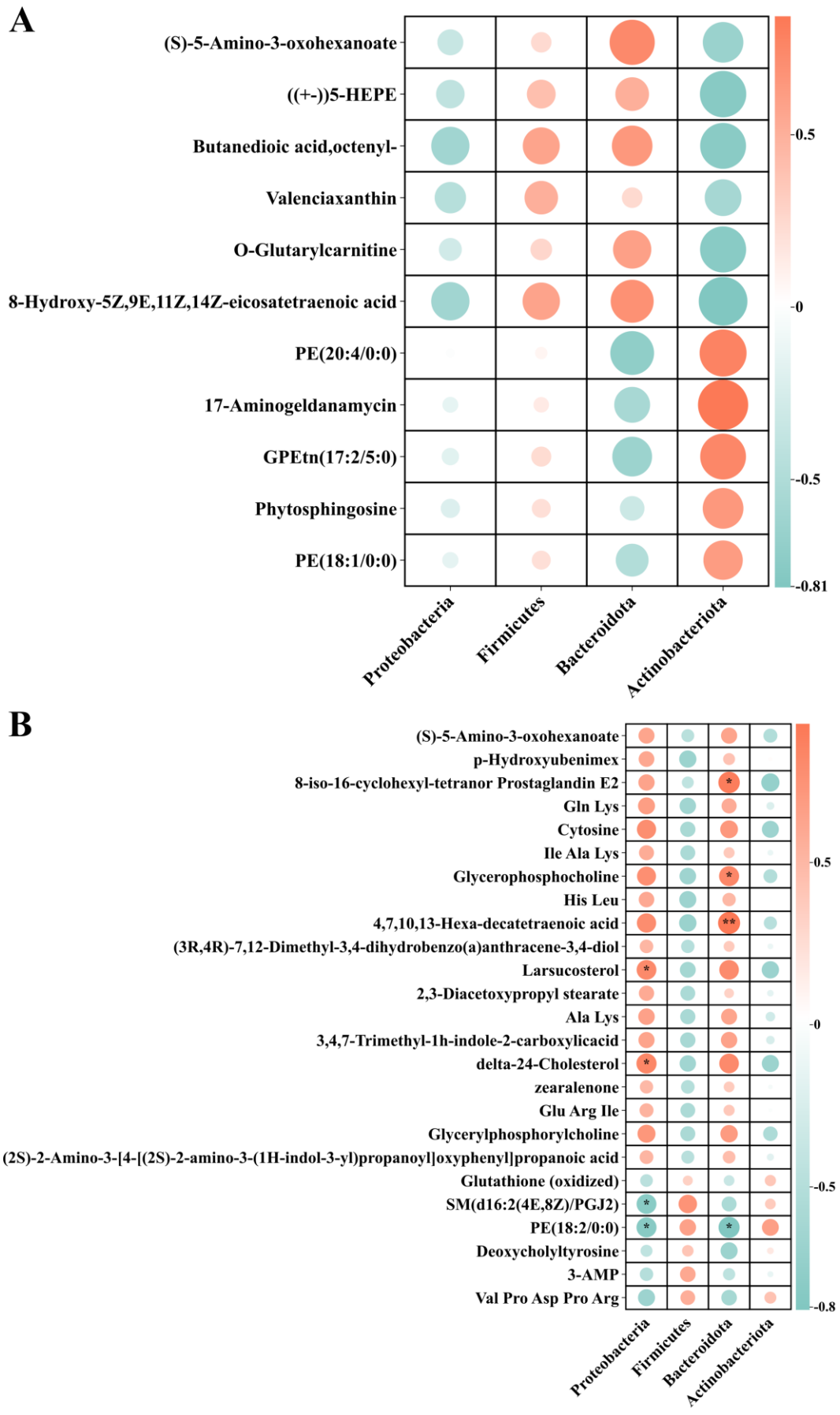


Figure 3.8. Correlation between gut microbiota and gut differentially expressed

metabolites (DEMs) after exposure to pristine and UV-aged polystyrene (PS) nanoplastics for 21 days. A, FP group; B, FA group.

3.4.3.4 Hepatic metabolomic alterations induced by pristine and UV-aged polystyrene (PS) nanoplastics and KEGG pathway analysis

As shown in Fig. 3.9, in the FA group, among the 95 DEMs, 70 were up-regulated and 25 were down-regulated, while only 27 DEMs (8 were up-regulated and 19 were down-regulated) changed in the FP group. In the male groups, a total of 67 DEMs (30 were up-regulated and 37 were down-regulated) changed after exposure to pristine PS nanoplastics, and there were 24 up-regulated and 40 down-regulated metabolites that changed in the MA group. Hierarchical clustering was performed to further screen for DEMs. UV-aged PS nanoplastics caused a higher number of DEMs in the female exposure group than pristine nanoplastics (Fig. S3.4).

Among all treatment groups, the FA group had the highest number of DEMs, suggesting female zebrafish exhibited the most pronounced metabolomic disturbance following exposure to UV-aged PS nanoplastics. This pattern suggests that both biological sex and particle aging status influenced PS-nanoplastic-induced stress-related metabolic responses. In addition, most of the DEMs in the FA group and the MA group belonged to lipids, including glycerophospholipids, fatty acyls, steroids and steroid derivatives and prenol lipids (Fig. 3.10). Glycerophospholipids are the main components of biological membranes and precursors to lipid mediators, associated with numerous metabolic diseases (Xu et al., 2024); fatty acyls are important sources of energy and play a pivotal role in lipid composition (Xicoy et al., 2019); and steroid hormones play a regulatory role in regulating lipid metabolism and inflammation (Liang et al., 2022). The changes in these metabolites suggested potential dysregulation in lipid metabolism induced by UV-aged PS nanoplastics. Impacts on hepatic lipid metabolism were previously demonstrated as a component of hepatotoxicity of micro- and nanoplastics (Luan et al., 2024; Xu et al., 2024; Zhao et al., 2020a). The disruption of lipid metabolism can be induced by oxidative stress (Ito et al., 2019), which was observed in the aged PS nanoplastic treatment groups in our study (Fig. 3.3). Thus, the metabolome analysis provides further evidence that exposure

to UV-aged PS nanoplastics could result in metabolic disorders, particularly affecting lipid metabolism.

In the female groups, pristine PS nanoplastics altered carbohydrate metabolism and amino acid metabolism; while UV-aged PS nanoplastics altered more pathways, including membrane transport, digestive system, carbohydrate metabolism, endocrine system, amino acid metabolism, metabolism of cofactors and vitamins, and signal transduction (Fig. 3.9 and S3.6). In the male groups, pristine and UV-aged PS nanoplastics both altered global and overview maps, biosynthesis of other secondary metabolites, amino acid metabolism, and nucleotide metabolism.

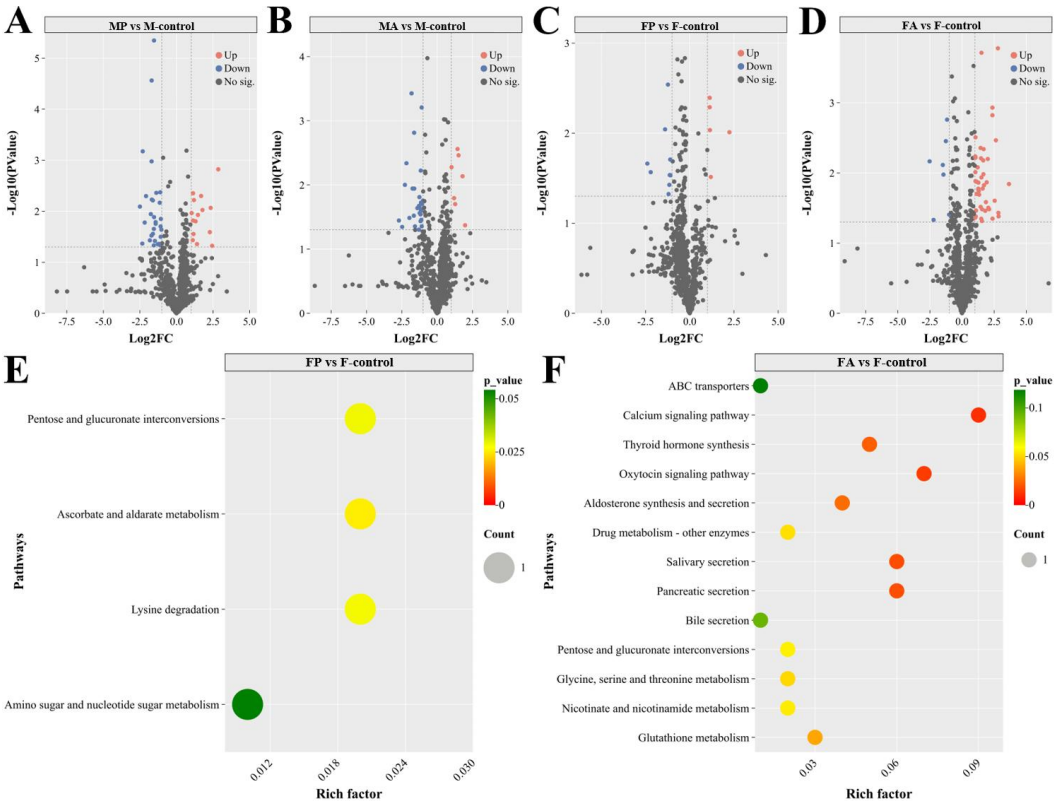


Figure 3.9. Volcano plot (A-D) and KEGG pathway enrichment (E and F) in zebrafish liver after exposure to pristine and UV-aged polystyrene (PS) nanoplastics for 21 days. A, MP group; B, MA group; C, FP group; D, FA group; E, FP group; F, FA group.

Micro- and nanoplastics 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 (Brun et al., 2019; Chen et al., 2022; Ye et al., 2021; Yu

et al., 2023). Specifically, our results showed that pristine and UV-aged PS nanoplastics both influenced amino acids metabolism and carbohydrate metabolism in both genders, which are basic biological processes for maintaining liver function and overall metabolic homeostasis (Xi et al., 2023; Zhang et al., 2022b). Among differential amino acids, tryptophan and phenylalanine are related to immunity and stress response (Salamanca et al., 2020). Choi et al. (2024) also found the exposure to polyethylene terephthalate microplastics 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.

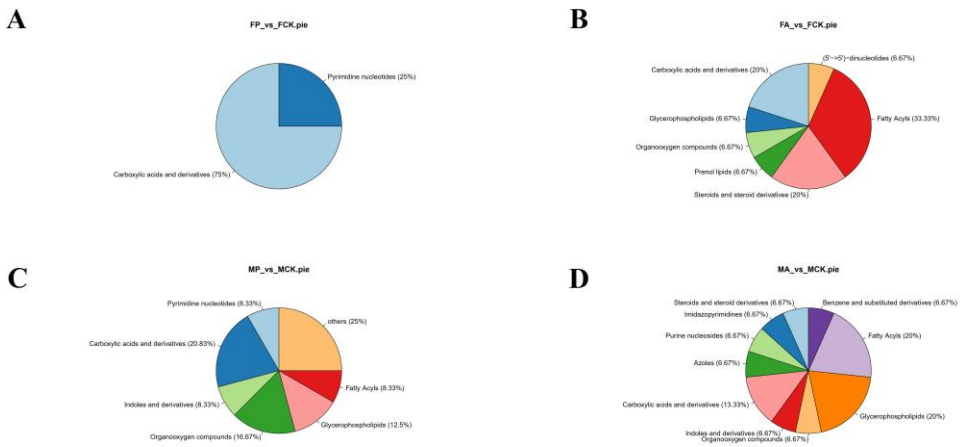


Figure 3.10. Classification of liver differentially expressed metabolites (DEMs) after pristine and UV-aged polystyrene (PS) nanoplastic exposure, A, FP group; B, FA group; C, MP group; D, MA group.

In addition, there were some uniquely influenced metabolic pathways (e.g. membrane transport, digestive system, endocrine system) in the FA group compared to the F-control and the MA group, indicating that exposure to pristine and UV-aged PS nanoplastics may disrupt different basic metabolic mechanisms as well as gender-specific impacts of UV-aged PS nanoplastics. In our study, betaine, enriched in the ABC transporter pathway, was down-regulated in the FA group. This indicated that UV-aged PS nanoplastics might disrupt the balance of the substrate transport system and affect

energy metabolism (Andolfo et al., 2015), which is consistent with a previous study (Tao et al., 2024). Meanwhile, betaine has strong antioxidant properties and plays an important role in resisting oxidative damage (Annunziata et al., 2019). 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 (Hou et al., 2024; Mi et al., 2024). Thus, the effects of UV-aged PS nanoplastics on the digestive system indicated potential gut function disorder in female zebrafish.

3.4.3.5 Correlation between influenced gut microbiota and liver differentially expressed metabolites (DEMs)

To further explore whether gut microbiota disturbance was associated with hepatic metabolic alteration, correlation analysis was performed between altered gut bacterial taxa and liver DEMs. In the FP group, relatively limited associations were observed. Bacteroidota was positively correlated with GPSer (22:6/18:0), while Actinobacteriota was positively correlated with GPSer (22:6/18:0) and GPCho (17:1/17:1) (Fig. 3.11A). Because GPSer and GPCho are glycerophospholipids, these associations suggest that pristine PS nanoplastic exposure may be linked to changes in hepatic membrane lipid-related metabolism.

In contrast, the FA group showed a more complex correlation pattern. Proteobacteria, Firmicutes and Bacteroidota were positively correlated with multiple hepatic DEMs, including lipid-, amino acid- and carbohydrate-related metabolites (Fig. 3.11B). Lipid metabolites accounted for the largest proportion of these associations, including glycerophospholipids, fatty acyls, steroids and steroid derivatives, and prenol lipids. These results suggest that UV-aged PS nanoplastic exposure was associated with a broader gut microbiota-liver metabolite response in female zebrafish, particularly involving lipid metabolism.

In male zebrafish, fewer microbiota-liver metabolite associations were detected. In the MP group, only Bacteroidota was positively correlated with organooxygen

compounds and lipid metabolites (Fig. 3.12A). In the MA group, Proteobacteria was positively correlated with lipid metabolites and negatively correlated with D-valine (Fig. 3.12B). Compared with the male groups and the FP group, the FA group therefore showed the most extensive associations between gut microbiota and hepatic DEMs.

These findings provide associative evidence linking gut microbiota dysbiosis with hepatic metabolic disturbance after UV-aged PS nanoplastic exposure. 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 (Chen et al., 2020). Several studies have demonstrated an association between metabolic disorders and gut microbiota dysbiosis after exposure to micro- and nanoplastics (Jing et al., 2022; Zhu et al., 2024), consistent with the results of our study. It is worth noting that, compared to the FP group, a more complicated association between gut microbiota and hepatic differential metabolites was observed in the FA group. Certain gut microbiota exhibited a significant correlation with lipids, especially glycerophospholipids (GPSer, GPCho, PC), amino acids and carbohydrates, indicating an intricate interplay between gut microbiota and liver glycolipid metabolism. These results suggest that UV-aged PS nanoplastics may strengthen the association between gut microbiota disturbance and hepatic glycolipid metabolism in female zebrafish through a potential gut-liver-axis-related stress-response pathway.

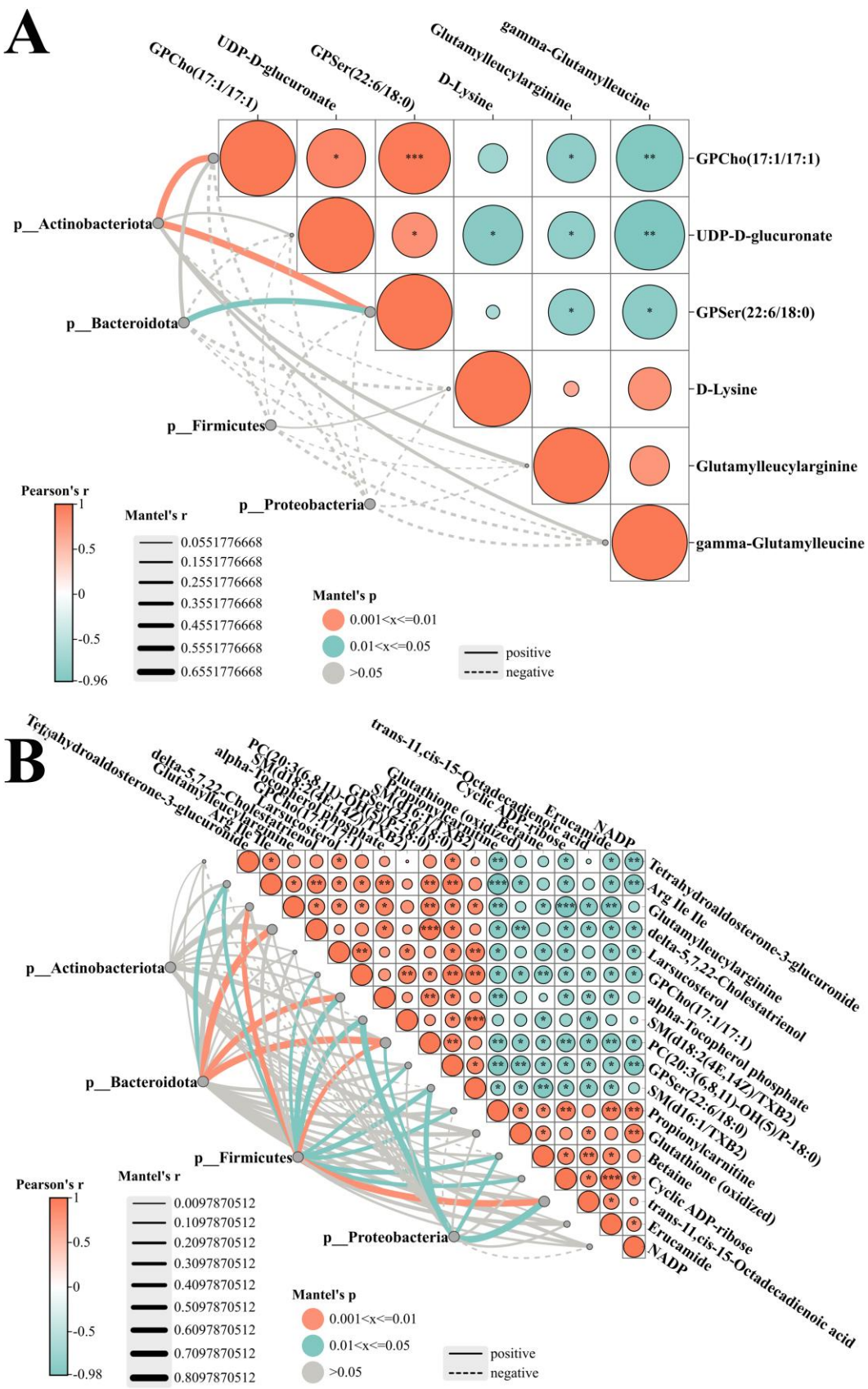


Figure 3.11. Correlation between gut microbiota and liver differentially expressed metabolites (DEMs) after exposure to pristine and UV-aged polystyrene (PS) nanoplastics for 21 days. A, FP group; B, FA group.

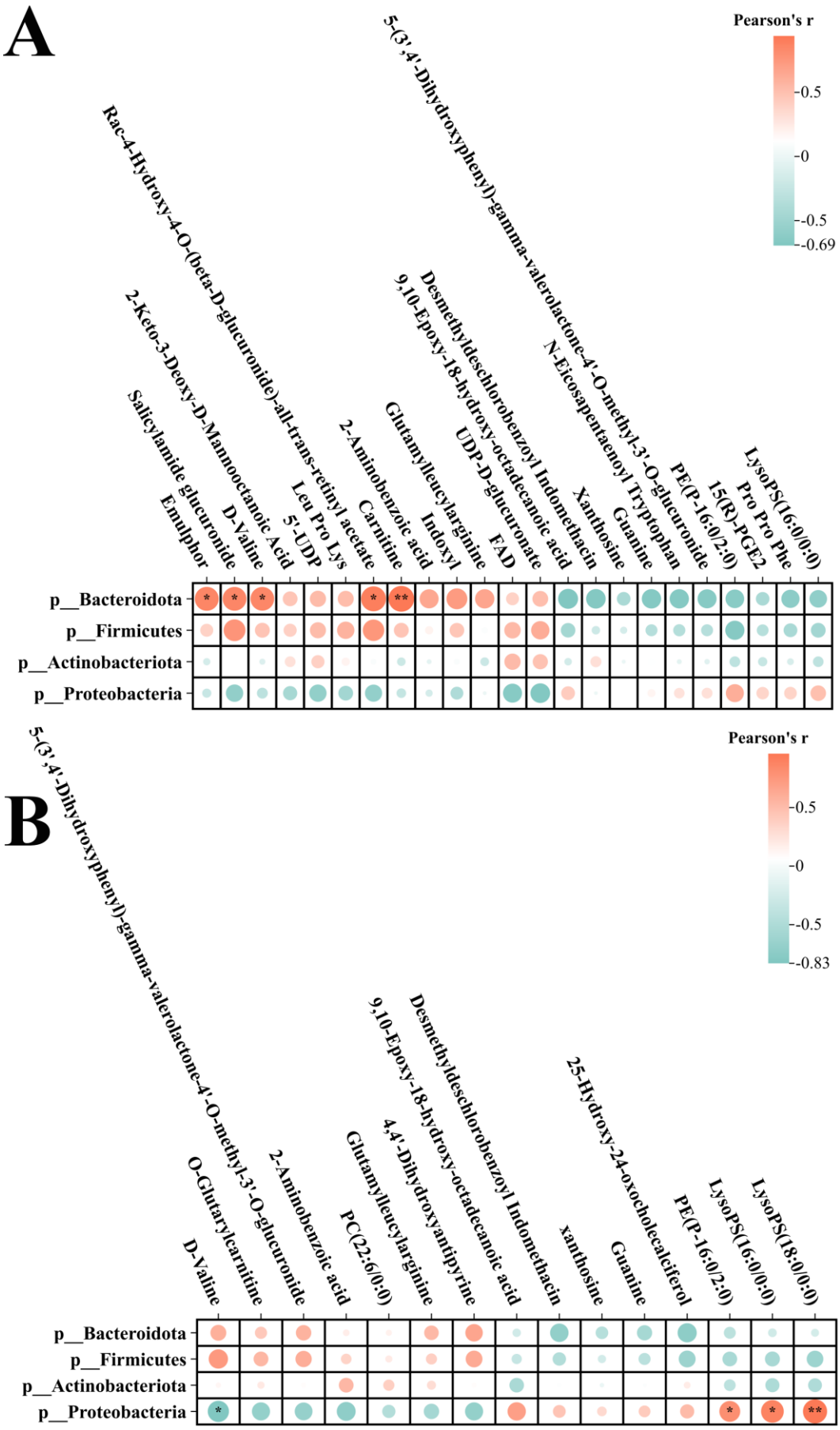


Figure 3.12. Correlation between gut microbiota and liver differentially expressed

metabolites (DEMs) of male zebrafish after exposure to pristine and UV-aged polystyrene (PS) nanoplastics for 21 days. A, MP group; B, MA group.

3.4.3.6 Correlation between influenced gut and liver lipid metabolites in the FA group and potential stress-related mechanism of UV-aged polystyrene (PS) nanoplastics 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. 3.13A).

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. It has previously been shown that microplastics can disturb glycerophospholipid and lipid metabolism in zebrafish (Zhao et al., 2020b).

Because female zebrafish exposed to UV-aged PS nanoplastics showed more pronounced alterations in gut microbiota and metabolism than those exposed to pristine particles, a conceptual model was developed to summarize the principal stress-related responses observed in this study. The present findings suggest that gut microbiota dysbiosis, glycolipid- and amino acid-related metabolic disturbance, and oxidative stress are key stress-related responses associated with UV-aged PS nanoplastic exposure in female zebrafish (Fig. 3.13B).

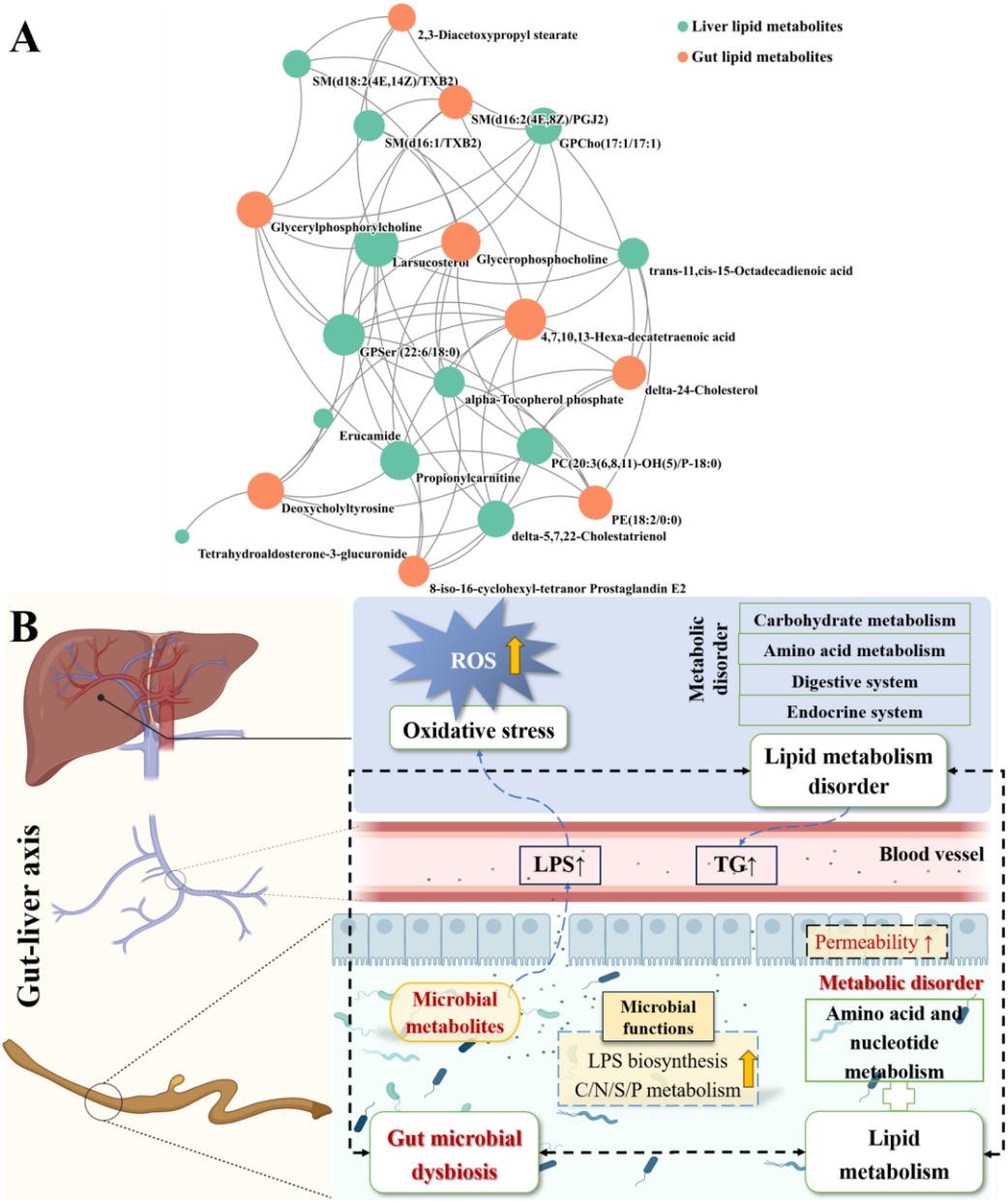


Figure 3.13. A, relevance network between gut lipid metabolites and liver lipid metabolites in the FA group. B, conceptual model summarizing the major stress-related responses associated with UV-aged polystyrene (PS) nanoplastic exposure in female zebrafish through the gut-liver axis. Solid boxes represent findings directly supported by the present study, whereas dashed arrows and boxes indicate proposed biological links inferred from the data. The model integrates gut microbiota dysbiosis, altered microbial functions, increased lipopolysaccharide (LPS)-related responses, metabolic disturbance and oxidative stress.

3.4.4 Environmental implications

Considering that micro- and nanoplastics in the environment inevitably undergo aging processes, the biological effects derived from aged micro- and nanoplastics on the freshwater ecosystem should be carefully considered. Our study provides new knowledge on the stress-related effects of pristine and UV-aged nanoplastics on freshwater organisms. Results revealed that UV photodegradation enhanced the stress-related biological responses of PS nanoplastics to zebrafish of both genders, while gut health and liver metabolism of female zebrafish are more affected by UV-aged PS nanoplastics than males. The main stress-related mechanisms of PS nanoplastics to zebrafish are gut microbiota dysbiosis, disruption to lipid and amino acids metabolism and oxidative stress. In addition, changed physicochemical properties through the aging process may influence the interaction of micro- and nanoplastics with other environmental pollutants, leading to more severe outcomes (Bhagat et al., 2022; Huang et al., 2021; Jiang et al., 2022). Our results emphasize that, when evaluating the biological effects and risks of micro- and nanoplastics, the impact of aging processes should be considered.

3.4.5 Limitations

This study provides novel insights into the stress-related mechanisms of pristine and UV-aged PS nanoplastics 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 UV-aged nanoplastics to freshwater ecosystems. This chapter has several limitations. First, the experimental particles were 500 nm PS nanoplastics, which fall within the nanoplastic size range and represent only one polymer type, one spherical particle form and one particle size. The results therefore cannot be generalised to larger PS microplastics, irregular fragments, fibres or other polymer types. Second, the UV aging treatment used UVC irradiation at 254 nm for six months. This provided a controlled accelerated photooxidation scenario, but it does not fully represent natural solar weathering, which is dominated by UVA and UVB radiation and occurs together with temperature fluctuation, mechanical abrasion, dissolved organic matter interactions and biofilm colonisation. Third, biological aging through biofilm formation

was not included, although biofilms are likely to alter particle density, aggregation, surface chemistry, microbial exposure and organismal interactions in natural freshwater systems. Fourth, only one exposure concentration was used, so concentration-response relationships could not be established. The concentration of 2.5 mg/L should be interpreted as a high laboratory exposure concentration rather than a typical environmental level. Fifth, sampling was conducted at a single terminal time point after 21 days, which prevents assessment of temporal dynamics, adaptation or recovery. Finally, the proposed gut-liver mechanism is based on associations among gut microbiota, serum indicators, oxidative stress and metabolomic profiles. These omics-based correlations do not prove causality, and direct validation of gut barrier function, inflammatory signalling, microbial translocation and targeted metabolic pathways would be required to confirm the mechanism.

3.5 Conclusions

This study showed that UV aging modified the physicochemical properties and biological effects of 500 nm PS nanoplastics in adult zebrafish. Compared with pristine PS nanoplastics, UV-aged PS nanoplastics induced stronger stress-related responses, including increased IgM, LPS and TG levels and enhanced hepatic oxidative stress. These responses may be partly associated with UV-induced changes in particle surface properties, particularly increased surface roughness and the formation of oxygen-containing functional groups. Female zebrafish showed broader stress-related responses to UV-aged PS nanoplastics than males, especially in relation to gut microbiota composition and gut-liver metabolic profiles. Both pristine and UV-aged PS nanoplastics were associated with gut microbiota dysbiosis and hepatic metabolic disturbance. However, UV-aged PS nanoplastics induced more pronounced changes in females, including an increased relative abundance of Proteobacteria and altered metabolic pathways related to lipid, amino acid and carbohydrate metabolism in the gut and liver. Correlation analysis further indicated significant associations among gut microbiota, gut metabolites and liver metabolites, suggesting a potential gut-liver-axis-related stress-response pattern in female zebrafish exposed to UV-aged PS nanoplastics. Overall, this

chapter demonstrates that UV aging can modify PS-nanoplastic-induced stress-related biological responses in adult zebrafish and highlights the need to consider particle aging status and biological sex when assessing the ecological risks of nanoplastics in freshwater systems.

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Chapter 4. Mechanical weathering of polystyrene microplastics intensifies biological stress but increases ecosystem multifunctionality in a simplified freshwater-sediment microcosm

4.1 Abstract

Microplastics are ubiquitous pollutants in freshwater ecosystems, yet their impacts on freshwater-sediment ecosystems remain poorly understood. Here, we investigated how pristine and mechanically weathered polystyrene (PS) microplastics influence organisms, sediment properties, and overall ecosystem multifunctionality (EMF) within an integrated freshwater-sediment microcosm containing the rooted macrophyte *Vallisneria spiralis*, the benthic snail *Lymnaea stagnalis*, and sediments. Results showed that mechanical weathering transformed the originally smooth spherical PS microplastics into particles with rougher and more irregular surfaces. Weathered PS microplastic induced stronger biological stress in both plants and snails, reflected in higher integrated biomarker response (IBR) scores. Weathered PS microplastics reduced chlorophyll a and carotenoid content and also altered plant nutrient content, indicating potential impaired photosynthesis and altered carbon metabolism. In snails, weathered PS microplastics triggered oxidative stress and significantly increased caspase-3 activity, suggesting potential elevated apoptotic responses and ionic regulation demands. Despite organism-level stress, both pristine and weathered PS microplastics increased EMF. This increase was primarily driven by increased dissolved organic carbon, elevated β-glucosidase activity, and intensified microbial respiration, reflecting short-term stimulation of sediment carbon processing. These results reveal a potential ecological trade-off between organismal stress and sediment-associated functional performance. Short-term functional stability was maintained through accelerated microbial metabolism, which may mask underlying stress responses in plants and snails. Our findings suggest that microplastic weathering can amplify biological stress while simultaneously promoting sediment-associated functions, highlighting that apparent short-term stability may entail long-term ecological risk. Integrating mechanically weathered microplastics with multi-species

ecosystem assessments provides a more comprehensive framework for evaluating the ecological risks of microplastic contamination.

4.2 Introduction

Microplastics have emerged as persistent and pervasive contaminants within aquatic environments, including rivers, lakes and sediments (Ahmed et al., 2021). High concentrations of microplastics have been extensively documented in aquatic systems globally with recent research focus shifting from oceans to freshwater and sediment environments (Yang et al., 2021a; Zhang et al., 2022a). Sediments in particular act as both accumulation zones and secondary sources, mediating prolonged interactions between microplastics, organisms and geochemical processes (Peng et al., 2017). Globally, reported microplastic abundances in freshwater sediments ranged from dozens to tens of thousands per kilogram. The highest recorded concentration was 335,714 items per kilogram, found in sediment from Lake Huron in Canada (Belontz et al., 2022).

Microplastics in freshwater sediments can result in direct and indirect impacts on element cycling, ultimately cascading into a suite of negative effects on organisms in the freshwater-sediment ecosystem (Berlino et al., 2021; Li et al., 2023; Ma et al., 2019). Furthermore, microplastics can alter physicochemical properties of sediments, the activities of microbes and benthic organisms, functional enzyme activities, and the expression of genes involved in nutrient metabolism, which may subsequently impact elemental cycling (Li et al., 2022; Yao et al., 2023; Yin et al., 2023). In addition, microplastics can have adverse impacts on the health of organisms in the freshwater-sediment ecosystem, including growth, reproduction, digestion, metabolism, survival, and the diversity of benthic fauna (Darabi et al., 2021).

Despite growing awareness, major gaps remain in our understanding of how microplastics impact interlinked ecological compartments, not simply individual species in isolation, but the combined dynamics of sediment, rooted aquatic plants, benthic invertebrates and overlying water. Rooted macrophytes and benthic snails play central roles in freshwater nutrient cycling, organic matter turnover and trophic coupling (Lv et al., 2022; Morini et al., 2024), yet their responses to microplastic exposure are rarely

integrated in a common experimental framework. Moreover, environmental weathering of microplastics is a continuous process involving mechanical abrasion, photochemical oxidation, chemical transformation, and biological degradation, which collectively modify particle morphology, surface chemistry, functional groups, hydrophobicity, and the release of polymer-derived constituents, thereby altering the environmental behaviour and biological effects of microplastics (Karlsson et al., 2018; Mao et al., 2020; Pfohl et al., 2022; Shi et al., 2024). While the weathering of microplastics through accelerated oxidation under laboratory conditions has been extensively investigated, mechanical abrasion remains comparatively understudied. In aquatic environments, mechanical abrasion occurred through wind-driven motion, water turbulence (including waves and tidal actions), and abrasion from sand, particularly in sediment-rich area (Zhang et al., 2024). Weathered microplastics may exhibit different ecological effects relative to pristine microplastics, but few studies have compared their effects in structurally complex microcosms. From an ecosystem-function perspective, rather than focusing solely on mortality or individual biochemical endpoints, it is increasingly important to adopt ecosystem multifunctionality (EMF) paradigms that capture the simultaneous performance of multiple processes, such as nutrient cycling, organic matter decomposition and carbon sequestration (Delgado-Baquerizo et al., 2016). Linking organism-level stress responses (e.g., oxidative stress, apoptosis) to sediment functionality and EMF provide a more holistic picture of ecosystem resilience or vulnerability under microplastic stress.

In this study, we established a simplified freshwater-sediment microcosm that integrates sediment, water, the rooted macrophyte *Vallisneria spiralis* and the freshwater snail *Lymnaea stagnalis*. We compared the impacts of pristine and mechanically weathered polystyrene (PS) microplastics on these interconnected compartments. Our objectives were: (i) to evaluate how mechanical weathering modifies the surface morphology, roughness, irregularity and approximate size distribution of PS microplastics; (ii) to evaluate how mechanical weathering modifies the biological (plants and snails) and sediment microbial/biogeochemical responses to PS microplastics; and (iii) to assess how these responses influence EMF. Based on the distinction between

stress-related responses and toxicity-related adverse outcomes defined in Chapter 1, this chapter examines both organismal stress responses and sediment-associated functional responses in a simplified freshwater-sediment microcosm. The first hypothesis was that pristine and mechanically weathered PS microplastics would induce stress-related responses in the submerged macrophyte *V. spiralis* and the freshwater snail *L. stagnalis*. This was assessed using plant pigment content, plant oxidative-stress biomarkers and snail enzyme activities. The second hypothesis was that PS microplastics would alter sediment-associated functional processes, including sediment environmental properties, extracellular enzyme activities, respiration and EMF. The third hypothesis was that mechanically weathered PS microplastics would induce stronger responses than pristine PS microplastics because physical weathering can alter particle morphology, roughness and sediment interaction. Changes in pigment content, caspase-3 activity were interpreted cautiously as potential adverse responses.

4.3 Materials and methods

The methodological design of this chapter was structured to evaluate organismal stress responses and functional responses in a simplified freshwater-sediment microcosm. First, pristine and mechanically weathered PS microplastics were prepared and characterised to compare the effects of pristine and mechanically weathered particles. Second, a laboratory freshwater-sediment microcosm containing artificial sediment, overlying water, *V. spiralis* and *L. stagnalis* was established to represent key interacting compartments under controlled conditions. Third, plant pigments, oxidative-stress biomarkers, nutrient-related indicators and snail enzyme activities were measured to assess organismal stress responses. Fourth, water and sediment physicochemical properties, sediment enzyme activities, respiration rate and EMF were measured to assess functional responses.

4.3.1 Sediment preparation, test organisms, and microplastics

The submerged plant *V. spiralis* was obtained from Biotope Aquatics Ltd. (United

Kingdom) and freshwater snails (*L. stagnalis*) were purchased from Blades Biological Ltd. (United Kingdom). PS is one of the most common types of microplastics found in freshwater sediments (Di & Wang, 2018). Pristine PS microplastics were sourced from Ningbo Chuangjin Import and Export Co., Ltd. Mechanical fragmentation has been used in previous studies to simulate physical weathering processes experienced by microplastics under environmental abrasion, including crushing and ball-milling treatments (Yan et al., 2023). Weathered PS microplastics were produced by mechanically grinding the pristine particles using a stainless-steel grinder. Particle morphology was examined using scanning electron microscopy (SEM) to confirm the physical differences between pristine and mechanically weathered PS microplastics. To provide an approximate estimate of particle-size distribution after mechanical weathering, SEM images were analysed using ImageJ. Randomly selected particles were measured from representative SEM fields, and particle size was expressed as equivalent circular diameter. Because mechanically weathered particles were irregular in shape, these measurements were used as approximate size descriptors rather than exact spherical diameters.

Artificial sediment was prepared according to OECD Guideline 218 (OECD, 2023) using sphagnum peat (4-5%), quartz sand (75-76%), kaolinite clay (20%), calcium carbonate (0.05-0.1%) and deionised water. The peat was air-dried, finely ground and suspended in deionised water. Its pH was adjusted to 5.5 ± 0.5 with $CaCO_3$, followed by conditioning for at least two days at 20 ± 2 °C under gentle stirring. The conditioned peat suspension was then mixed with quartz sand and kaolinite clay, and water was added to achieve a final moisture content of 30-50% of the dry sediment weight. The final pH was adjusted to 6.5-7.5, with an organic carbon content of approximately 2 ± 0.5%. This standardised artificial sediment reduced variability but did not fully represent the complexity of natural sediments. The artificial sediment was not sterilised before use. The microbial community was established during the sediment-conditioning period, primarily through microbial colonisation associated with the sediment components. After conditioning, the sediment was thoroughly homogenised and divided among all experimental groups. Therefore, the sediment enzyme activities and respiration measured in this study reflect the activity of the microbial community established within the

experimental system rather than the complex indigenous microbiota of field-collected freshwater sediment.

4.3.2 Overall experimental design

A 5-week simplified laboratory freshwater-sediment microcosm experiment was established, containing an artificial sediment layer, overlying deionized water, freshwater plant *V. spiralis* and snail *L. stagnalis*. Although artificial sediment and deionised water were used, the system contained the major experimental compartments required to examine cross-compartment interactions: a sediment layer, overlying water, a rooted submerged macrophyte, and a benthic snail. This controlled design was selected to reduce variability associated with field-collected sediment and natural water chemistry, thereby allowing the effects of pristine and mechanically weathered PS microplastics to be compared under standardised conditions. However, because the system did not include natural sediment, natural water, or the full microbial and trophic complexity of freshwater ecosystems, the findings should be interpreted as mechanistic microcosm evidence rather than direct field simulation.

Microcosms were constructed in 3 L glass beakers and included 800 g of dried sediment in each glass beaker. Pristine and weathered PS microplastics were added into the sediment to obtain a concentration of 0.1% of the sediment dry weight (1 mg PS microplastics/g dry sediment) by thorough manual homogenization. This concentration is commonly used in sediment microcosm studies to evaluate biological and ecosystem-level responses under relatively high contamination scenarios (Jakubowska et al., 2020; Li et al., 2024; Yu et al., 2022a). The plants and snails were introduced into the system, and 2.8 L of deionized water was slowly added to each replicate. No external nutrient solution was added during the 5-week exposure. This design was selected to minimise confounding effects from added nutrients and to allow sediment-associated nutrient and carbon processes to respond to PS microplastic exposure and organismal activity. However, this also means that the microcosms likely represented nutrient-limited conditions rather than nutrient-replete natural freshwater conditions. Therefore, the responses of *V. spiralis*, *L. stagnalis* and sediment microbial processes may reflect the

combined influence of PS microplastic exposure and nutrient limitation. The results should consequently be interpreted as PS microplastic exposure effects under simplified nutrient-limited freshwater-sediment microcosm conditions, rather than as a direct simulation of natural freshwater ecosystems.

Treatments included control, pristine PS microplastic, and weathered PS microplastic groups. Each treatment consisted of five independent replicate glass beakers (n = 5). Each beaker contained approximately 800 g of artificial sediment, five healthy Vallisneria spiralis, and three Lymnaea stagnalis individuals. Each beaker was considered one independent experimental replicate for all subsequent analyses. After exposure, sediment and water samples were collected for physicochemical and enzymatic analyses, while plant and snail tissues were analysed using a suite of biochemical assays.

4.3.3 Water and sediment properties

Water and sediment properties were measured to evaluate whether PS microplastics altered the physicochemical and functional conditions of the microcosm. Dissolved oxygen, pH and conductivity of the overlying water were measured every five days using a HACH meter. Sediment pH was measured using a pH meter with a sediment-to-water ratio of 1:2.5 (w/v). Dissolved organic carbon (DOC), total carbon (TC) and total nitrogen (TN) content in sediment and TC, total organic carbon (TOC), total inorganic carbon (TIC) content in water were determined with TOC analyser (Elementar vario MICRO cube). β-1,4-glucosidase (BG), a key enzyme involved in sediment carbon cycling, and urease, commonly used as an indicator of nitrogen cycling (Liu et al., 2024b), were measured using commercial assay kits (Nanjing Jiancheng Bioengineering Institute, China). The respiration rate of sediment was determined following established protocols described in reference (Haney et al., 2008).

4.3.4 Plant analysis

Plant endpoints were measured to evaluate whether PS microplastics affected photosynthetic status, surface morphology, oxidative stress and nutrient-related responses

in *V. spiralis*.

4.3.4.1 Chloroplast pigments

Chlorophyll-a, chlorophyll-b and carotenoids were measured because they reflect photosynthetic pigment status and potential stress effects on photosynthesis. The chlorophyll contents of plants were extracted from 0.2 g of leaf using 15 mL of 95 % alcohol at room temperature. The concentrations of chlorophyll-a (Chl-a), chlorophyll-b (Chl-b), and carotenoids were determined spectrophotometrically at 665 nm, 649 nm, and 470 nm, respectively, and calculated as follows (Siegfried et al., 2017):

$$C_a = 13.95A_{665} - 6.88A_{649} \quad (4.1)$$

$$C_b = 24.96A_{649} - 7.32A_{665} \quad (4.2)$$

$$C_\chi = \frac{1000A_{470} - 2.05C_a - 114.8C_b}{245} \quad (4.3)$$

where C_a, C_b and C_χ are the concentration of Chl-a, Chl-b and carotene, respectively, A665, A649 and A470 are absorbances in the wavelength of 665 nm, 649 nm, and 470 nm, respectively, for chlorophyll extracts.

4.3.4.2 Scanning electron microscopy (SEM) observation

SEM observation was used to examine whether PS microplastics were associated with plant surfaces and whether visible surface changes occurred. The leaf and root samples were fixed in 2.5% glutaraldehyde, dehydrated in gradient concentrations of ethanol, coated with a layer of gold, and then the images were observed using SEM (Carl Zeiss EVO MA 15).

4.3.4.3 Oxidative stress markers

Total antioxidant capacity (T-AOC), superoxide dismutase (SOD) activity, and malondialdehyde (MDA) were measured to assess antioxidant capacity, antioxidant enzyme response and lipid peroxidation, respectively. Leaves were homogenized in 0.1 mol L^{-1} phosphate-buffered saline (PBS, pH 7.4), and the homogenates were centrifuged at 10,000 rpm for 15 minutes at 4 °C. The resulting supernatants were used to measure T-

AOC, SOD and MDA content using assay kits (Nanjing Jiancheng Bioengineering Institute, China). T-AOC was determined based on the ferric ion reducing antioxidant power (FRAP) assay. SOD activity was measured using the xanthine-xanthine oxidase method by quantifying the inhibition of superoxide anion-mediated nitrite formation. MDA content was determined using the thiobarbituric acid (TBA) method as an indicator of lipid peroxidation.

4.3.4.4 Nutrient content

Soluble sugar, starch and free amino acids were measured to evaluate nutrient and energy-related responses in plant leaves. Leaf soluble sugar, starch, and free amino acid contents were measured using commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's protocols. Briefly, approximately 0.1 g fresh leaf tissue was homogenized with the corresponding extraction solution, followed by centrifugation to obtain the supernatant for colorimetric determination. Soluble sugar and starch contents were determined by the anthrone colorimetric method (Zeng et al., 2022), while free amino acid measurements were performed with the ninhydrin method (Yemm et al., 1955).

4.3.5 Oxidative stress and enzyme activities in snails

Snail biochemical endpoints were measured to assess stress-related responses in *L. stagnalis* after PS microplastic exposure. The soft tissues of snails were collected because they integrate whole-organism physiological responses after contact with sediment, water and ingested particles. T-AOC, SOD and MDA were measured to assess antioxidant capacity, antioxidant enzyme response and lipid peroxidation. ATPase activity was measured as an indicator of energy-metabolism-related enzyme activity. AKP activity was used as an immune- and metabolism-related enzyme indicator, while AChE activity was measured as a neurophysiological enzyme response. Caspase-3 activity was measured as an apoptosis-related indicator. Snail soft tissues were homogenised in ice-cold PBS, centrifuged, and the supernatants were analysed using commercial assay kits according

to the manufacturer's protocols. The soft tissues of three snails were collected and homogenized in ice cold PBS solution using a glass homogenizer. T-AOC, SOD activity, MDA content, ATPase, alkaline phosphatase (AKP), acetylcholinesterase (AChE) and caspase-3 activities were measured by assay kits (Nanjing Jiancheng Bioengineering Institute, China). ATPase activity was determined by measuring the inorganic phosphate released during ATP hydrolysis. AKP activity was measured colorimetrically based on the hydrolysis of disodium phenyl phosphate. AChE activity was determined using a colorimetric method based on the hydrolysis of acetylthiocholine. Caspase-3 activity was determined using a colorimetric assay based on the cleavage of the chromogenic substrate Ac-DEVD-p-nitroanilide (Ac-DEVD-pNA), with the released p-nitroaniline quantified at 405 nm.

4.3.6 Integrated biomarker response version 2 (IBRv2) analysis and ecosystem multifunctionality (EMF)

IBRv2 analysis was conducted to integrate the indicator responses of plants and snails into composite indices, calculated separately for each treatment group. The plant-related indicators included Chl-a, carotenoids, SOD, MDA, soluble sugars, amino acids and starch, while snail related indicators included SOD, MDA, AChE, AKP, ATPase, and caspase-3 (Text S1).

Freshwater-sediment EMF was characterized to evaluate the simultaneous performance of multiple sediment-associated functions in the microcosm. In this chapter, EMF was calculated using seven sediment-related variables representing three functional domains: sediment chemical condition (pH), carbon availability and cycling (dissolved organic carbon, total carbon, β-1,4-glucosidase activity and respiration rate), and nitrogen availability and transformation (total nitrogen and urease activity). Z-scores were used to standardize each variable following the approach of Delgado-Baquerizo et al. (2016).

$$Z = \frac{X - \mu}{\sigma} \tag{4.4}$$

where X is the measured value of a given function, mean is the mean value across all treatments, and SD is the standard deviation across all treatments. The EMF index was

then calculated using the averaging approach, by taking the mean of the standardised values across all included functions for each replicate (Liu et al., 2024b). The EMF value is sensitive to the choice of included functions and their directional interpretation. Therefore, EMF was interpreted together with individual functional variables and organism-level biomarker responses rather than as a standalone indicator of ecosystem improvement.

4.3.7 Data analysis

The data are presented as mean ± standard deviation. Normality was assessed using the Shapiro-Wilk test, and homogeneity of variance was assessed using Levene's test. Normally distributed data with homogeneous variance were analysed using one-way ANOVA followed by Tukey's post hoc test. If the data were normally distributed but variance homogeneity was not met, Welch ANOVA followed by Games-Howell post hoc test was used. A significance threshold of $p < 0.05$ was applied. Exact p-values for the statistical comparisons shown in the Chapter 4 figures are provided in Table S4.1.

4.4 Results and discussion

4.4.1 Mechanical weathering process alters property of polystyrene (PS) microplastics

SEM images of pristine microplastics showed smooth surfaces, whereas mechanically weathered microplastics exhibited rougher surfaces and more irregular morphologies (Fig. 4.1). SEM-based particle size analysis showed that pristine and mechanically weathered PS microplastics had comparable particle size distributions, suggesting that mechanical weathering primarily altered surface morphology rather than causing substantial particle-size reduction (Table 4.1). These changes demonstrate that mechanical weathering altered the surface structure of PS microplastics, increasing surface roughness.

SEM observations revealed roughened and irregular particle surfaces after mechanical weathering, whereas FTIR spectra remained nearly identical between pristine

and weathered PS microplastics, indicating that mechanical weathering mainly altered the physical surface characteristics of the particles without substantially changing the chemical structure of the PS polymer. Yan et al. (2023) demonstrated that mechanical weathering substantially altered the surface morphology and physicochemical characteristics of PS, PP, and polyethylene terephthalate microplastics through free radical processes, increasing surface roughness. In our study, weathered PS microplastics induced higher oxidative and metabolic disturbances in organisms, underscoring the potential biological risks of weathered microplastics to ecosystem organisms. These findings align with previous study, reporting that weathering process could enhance interactions with living organisms and increase bioavailability and biological effects (Li et al., 2025). Moreover, these results indicate that mechanical fragmentation, though less studied than photochemical or oxidative aging, critically shapes microplastic biological effects in aquatic systems.

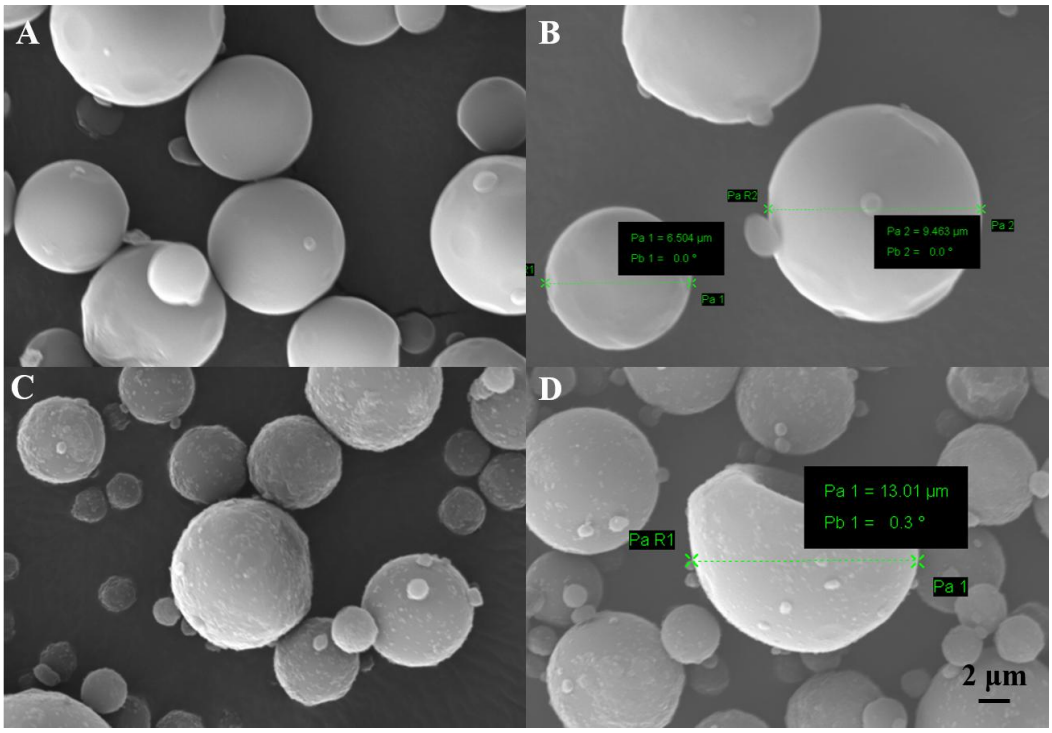


Figure 4.1. Scanning electron microscopy (SEM) images of pristine (A and B) and mechanically weathered (C and D) PS microplastics.

Although mechanical weathering is often reported to generate smaller plastic

fragments through fragmentation (Bhattacharjee et al., 2025; Arredondo-Navarro et al., 2026), no significant reduction in particle size was observed in the present study. Instead, mechanical weathering primarily altered the surface morphology of PS microspheres, producing rougher surfaces and irregular edges while maintaining a similar particle size distribution. This discrepancy is likely attributable to the relatively short weathering duration and the intrinsic properties of the commercial PS microspheres used in this study. Thus, changes in surface properties, including increased roughness and irregular morphology, are more likely responsible for the increased biological responses to weathered PS microplastics in this study.

Table 4.1. Particle size characteristics of pristine and mechanically weathered polystyrene (PS) microplastics determined from scanning electron microscopy (SEM) images.

Group	n	Mean ± SD (μm)	Median(μm)	Range(μm)
Pristine PS microplastics	107	6.32 ± 2.05	6.34	2.58-12.16
Mechanically weathered PS microplastics	111	6.14 ± 2.26	5.68	2.58-13.61

Statistical comparison: Welch's t-test, P = 0.534; Mann-Whitney U test, P = 0.344.

4.4.2 Impact of polystyrene (PS) microplastics on water and sediment properties

The pH and dissolved oxygen levels in all groups increased over time, although no significant difference was observed between the control and microplastic treatments after exposure (Fig. S4.1). A significant increase ($p < 0.05$) in conductivity was found in both pristine and weathered microplastic treatments compared to the control. Similarly, both TC and TOC concentrations in the overlying water increased significantly after PS

microplastic exposure (Fig. 4.2).

PS microplastics significantly altered sediment properties with pH, TC and DOC in both pristine and weathered microplastic groups being higher than that of the control (Fig. 4.3A-C). Urease activity decreased significantly in the weathered PS microplastic group, whereas BG activity increased in both pristine and weathered PS groups (Fig. 4.3D and E). Moreover, both pristine and weathered microplastics enhanced sediment respiration rates.

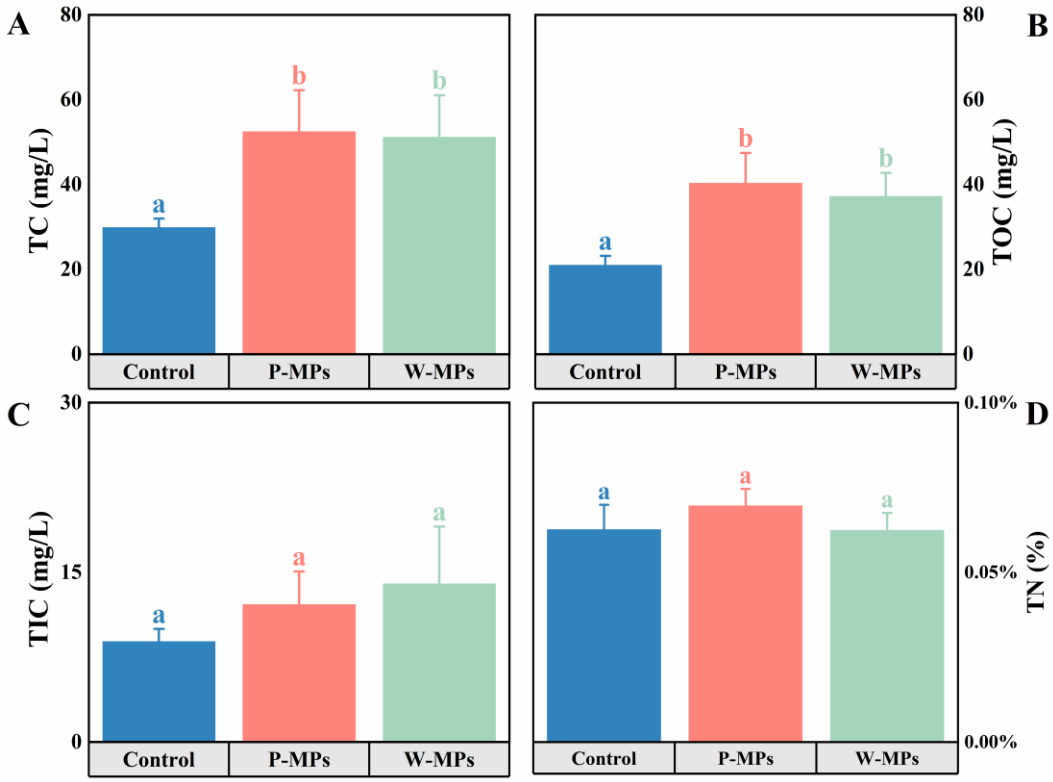


Figure 4.2. Carbon content in water and nitrogen content in sediment. A: total carbon in water; B: total organic carbon in water; C: total inorganic carbon in water; D: total nitrogen in sediment. P-MPs represent pristine microplastics and W-MPs represent weathered microplastics. Data represent mean ± SD. Different letters indicate significant differences between treatments ($p < 0.05$).

Both pristine and weathered PS microplastics significantly altered sediment properties and microbial processes (Fig. 4.3). Previous studies showed that microplastics caused different impacts on soil/sediment pH, depending on polymer type, concentration, shape, and size (Wang et al., 2022; Zhao et al., 2021). For example, Liu et al. (Liu et al.,

2024a) found that polyamide (PA) microplastics increased sediment pH at 0.1% and 1% concentrations (w/w), potentially due to their aging and degradation. Li et al. (Li et al., 2021) found that microplastics reduced pH in acid soils but increased pH in alkaline soils, suggesting that microplastic accumulation would induce deterioration of pH in soils. Microplastics can also indirectly influence sediment pH by affecting microbial activity (Zhou et al., 2021). As pH plays a key role in nutrient bioavailability and plant performance, such shifts may have broader ecosystem implications. Given that PS microplastics are primarily composed of carbon, their addition directly increased sediment TC content in this study. Generally, the carbon present in non-degradable microplastics consists of polymers that are difficult to degrade, however, previous studies have shown that although the impact on DOC content is smaller than that of degradable microplastics, non-degradable microplastics could still increase DOC content in soil/sediment, as observed in this study (Feng et al., 2024; Xiang et al., 2024). Furthermore, as the addition of microplastics has the potential to stimulate microbial activity, thereby promoting DOC accumulation in soil/sediment (de Souza Machado et al., 2018; Liu et al., 2017). In our study, the concurrent increase in TC and DOC (Fig. 4.3B-C) indicates greater carbon availability and accelerated microbial carbon processing, which is consistent with the elevated BG activity and respiration rates observed (Fig. 4.3E-F). These carbon-related processes were among the strongest positive contributors to EMF (Fig. 4.11), demonstrating that microplastic-driven shifts in sediment carbon turnover played a central role in the overall enhancement of ecosystem multifunctionality.

Enzymatic responses further demonstrated these alterations. Elevated BG activity and increased sediment respiration rates indicated enhanced microbial decomposition and carbon cycling (Gudmundsson et al., 2016; Wang et al., 2024). Several studies have shown that the addition of microplastics can increase soil respiration rate by stimulating microbial and enzymatic activities (Gao et al., 2024; Song et al., 2024; Wang et al., 2024). In addition, another study reported that the addition of microplastics increased respiration rate and microbial growth by increasing the pH and DOC content, with DOC being considered the source of soil microbial energy and nutrients (Gao et al., 2021). While most studies have focused on soils, relatively little is known about microplastic effects on

respiration in freshwater sediments. In contrast, urease activity was inhibited in the weathered PS microplastic group (Fig. 4.3D), suggesting an inhibition of nitrogen transformation processes by weathered PS microplastics. The decreased urease activity was potentially related to reduced sediment TN content (Li et al., 2022; Liu et al., 2024b). These contrasting effects imply that microplastics can shift sedimentary metabolic balance toward carbon cycling dominance. In addition, more attention should also be directed toward microbial processes in the sediment, including microbial community structure, gene expression, and nutrient cycling pathways, to better understand the underlying stress- related mechanisms of microplastic.

The observed enhancement in EMF under microplastic exposure likely reflects the combined effects of increased microbial activity and carbon substrate availability. Such short-term stimulation of biogeochemical functions can elevate multifunctionality indices even under biological stress.

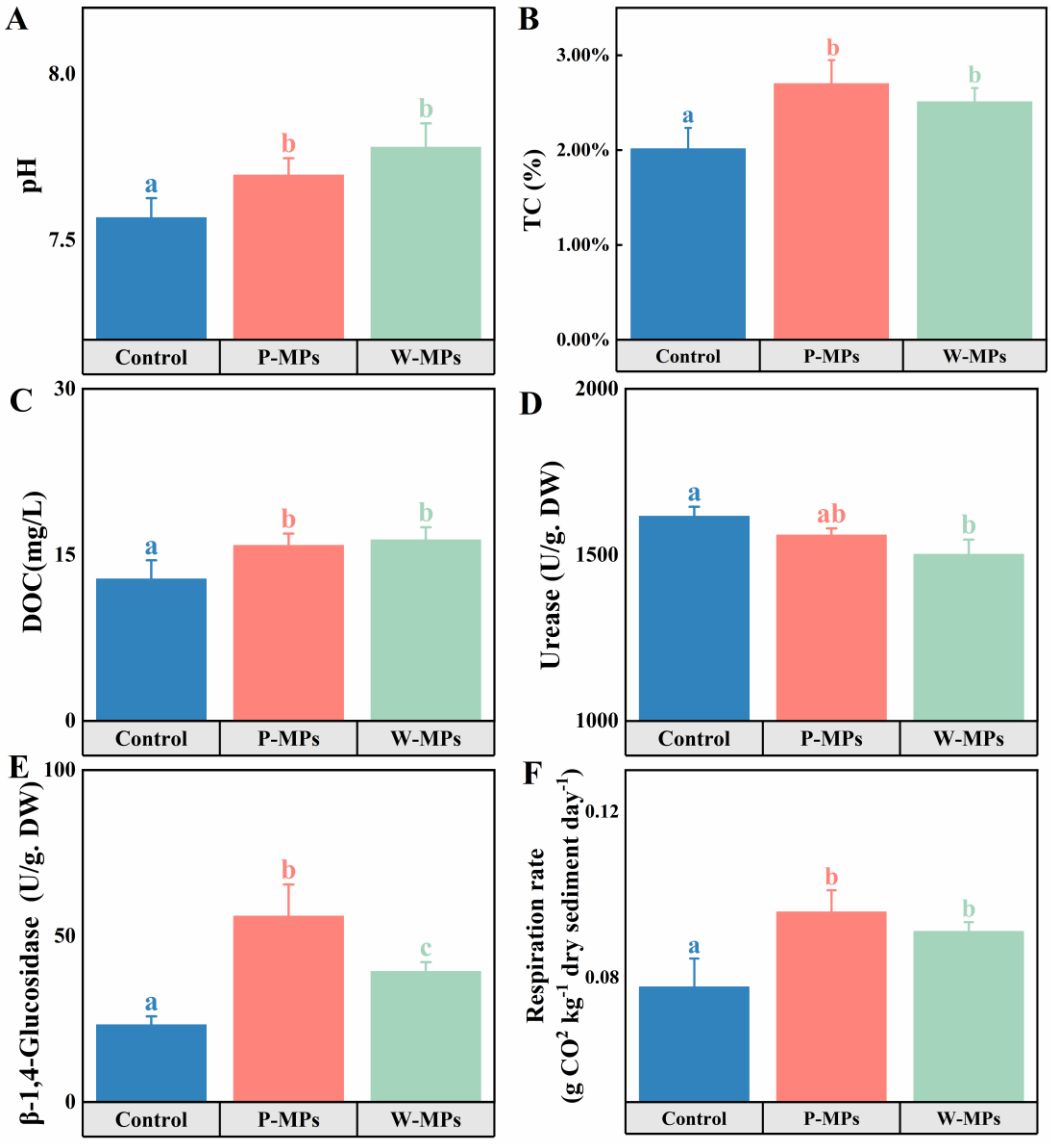


Figure 4.3. Alteration of sediment properties after exposure to pristine and weathered polystyrene (PS) microplastics. A: pH; B: total carbon (TC) content; C: dissolved organic carbon (DOC) content; D: urease activity; E: β-1,4-glucosidase (BG) activity; F: respiration rate. P-MPs represent pristine microplastics and W-MPs represent weathered microplastics. Data represent mean ± SD. Different letters indicate significant differences between treatments ($p < 0.05$).

4.4.3 Impact of polystyrene (PS) microplastics on plant *V. spiralis* and snail *L. stagnalis*

Pristine and weathered PS microplastics did not result in any visible plant damage or affect plant fresh weight, although weathered PS microplastics significantly reduced Chl-

a and carotenoid content, while pristine particles decreased carotenoid content (Fig. 4.4). SEM images revealed that weathered microplastics adhered to both leaves and roots of *V. spiralis* (Fig. 4.5 and 4.6), confirming direct physical interaction between microplastics and plant tissues.

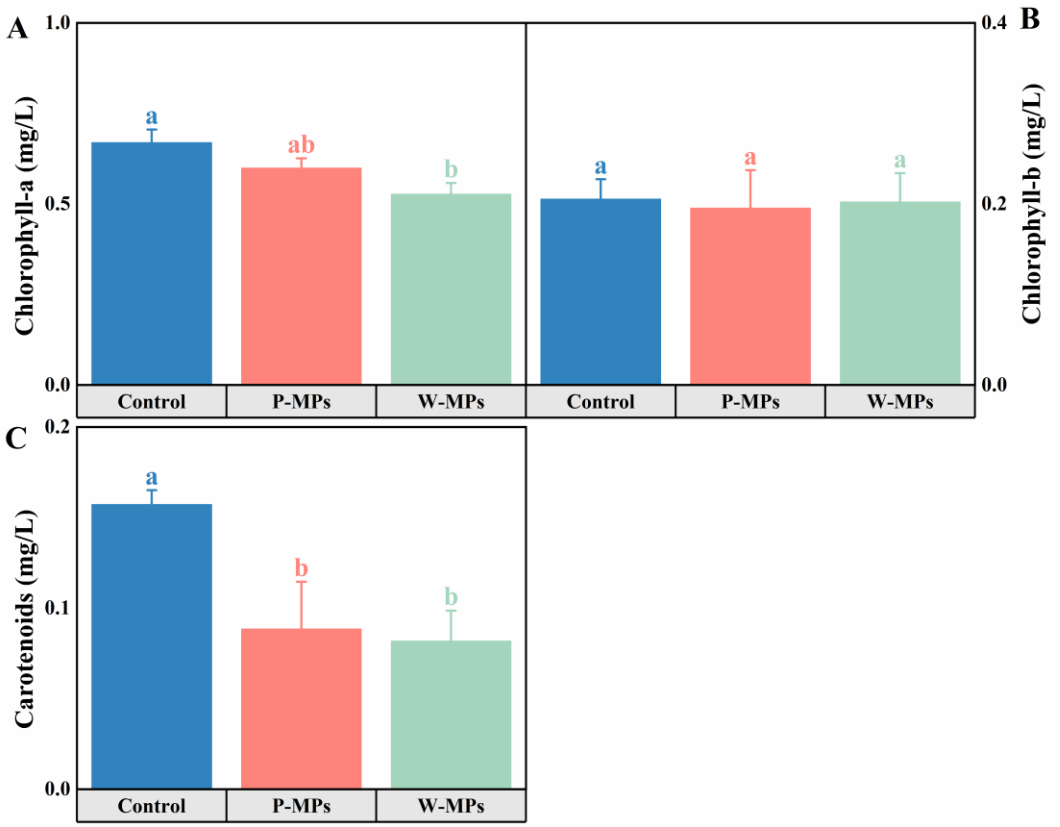


Figure 4.4. Chloroplast pigment level in plant leaves after exposure to pristine and weathered polystyrene (PS) microplastics. A: Chlorophyll-a; B: Chlorophyll-b; C: carotenoids. P-MPs represent pristine microplastics and W-MPs represent weathered microplastics. Data represent mean ± SD. Different letters indicate significant differences between treatments ($p < 0.05$).

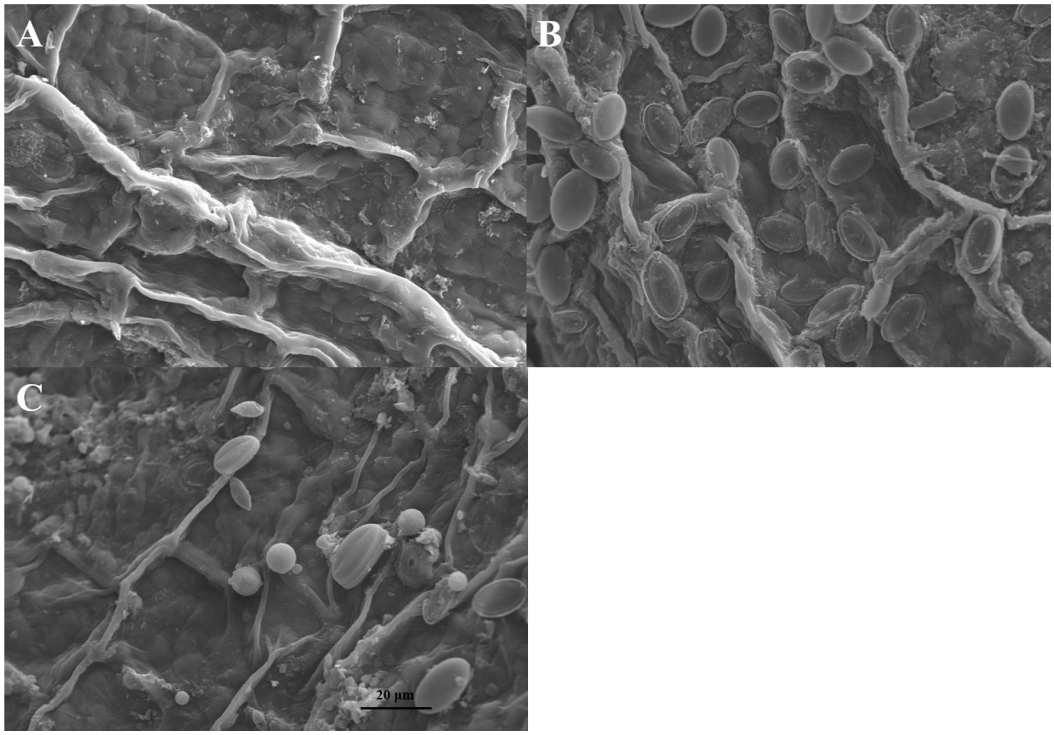


Figure 4.5. Scanning electron microscopy **(**SEM) images of plant leaves after exposure to pristine and weathered polystyrene (PS) microplastics. A: control group; B: pristine PS microplastic group; C: weathered PS microplastic group.

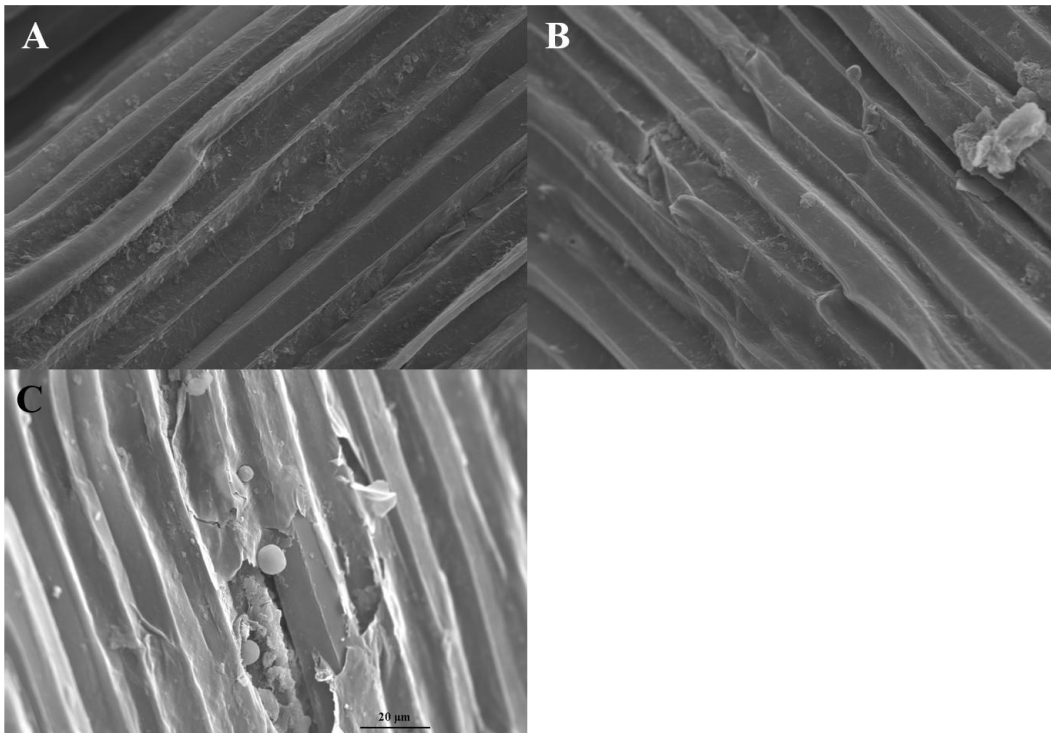


Figure 4.6. Scanning electron microscopy **(**SEM) images of plant roots after exposure to pristine and weathered polystyrene (PS) microplastics. A: control group; B: pristine PS microplastic group; C: weathered PS microplastic group.

In *V. spiralis*, weathered PS significantly increased SOD activity and MDA content, whereas T-AOC remained unchanged (Fig. 4.7). Weathered PS microplastics also increased soluble sugar and free amino acid levels while decreased starch content in the leaves (Fig. 4.8).

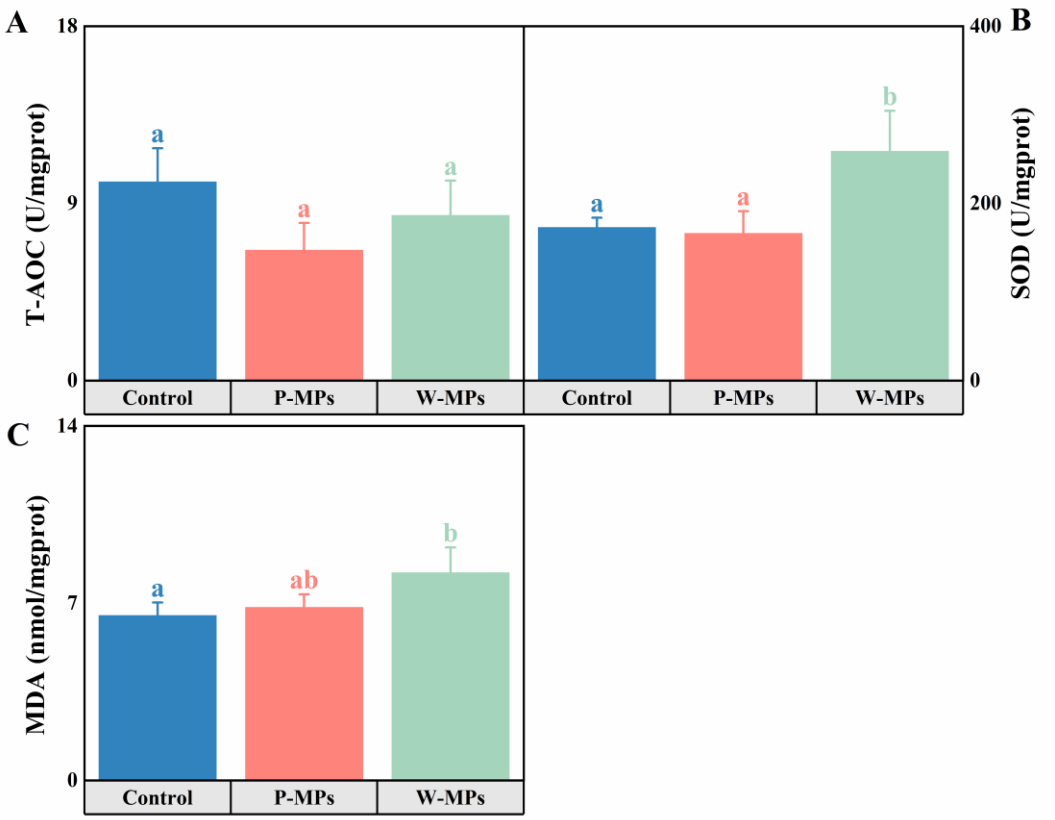


Figure 4.7. Oxidative stress in plant leaves after exposure to pristine and weathered polystyrene (PS) microplastics. A: Total antioxidant capacity (T-AOC) level; B: superoxide dismutase (SOD) activity; C: malondialdehyde (MDA) content. P-MPs represent pristine microplastics and W-MPs represent weathered microplastics. Data represent mean ± SD. Different letters indicate significant differences between treatments ($p < 0.05$).

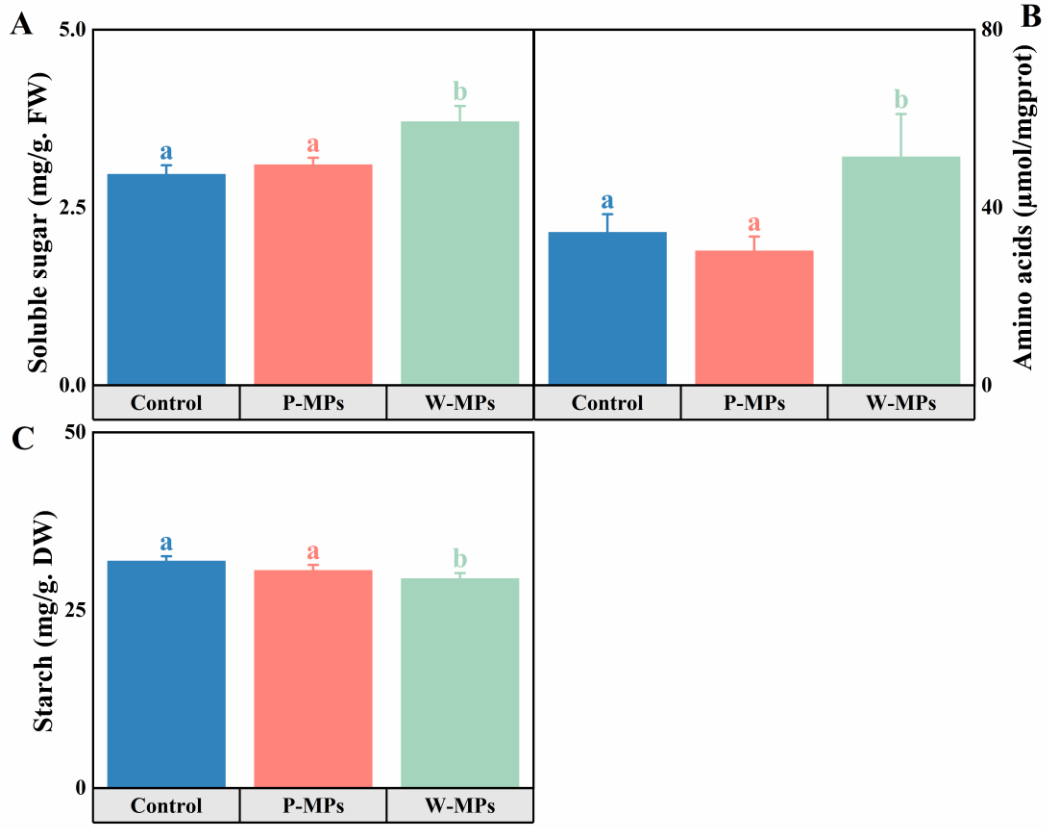


Figure 4.8. Nutrient content in plant leaves after exposure to pristine and weathered polystyrene (PS) microplastics. A: soluble sugar; B: free amino acids; C: starch. P-MPs represent pristine microplastics and W-MPs represent weathered microplastics. Data represent mean ± SD. Different letters indicate significant differences between treatments ($p < 0.05$).

In *L. stagnalis*, weathered PS microplastics significantly increased SOD activity, while pristine PS microplastics had no significant effects (Fig. 4.9). No significant change in T-AOC and MDA levels was found in all microplastic treatments. Caspase-3 activity was significantly promoted in the weathered PS microplastic group, whereas AChE and AKP activities remained unaffected. Weathered PS significantly increased ATPase activity (Fig. 4.10).

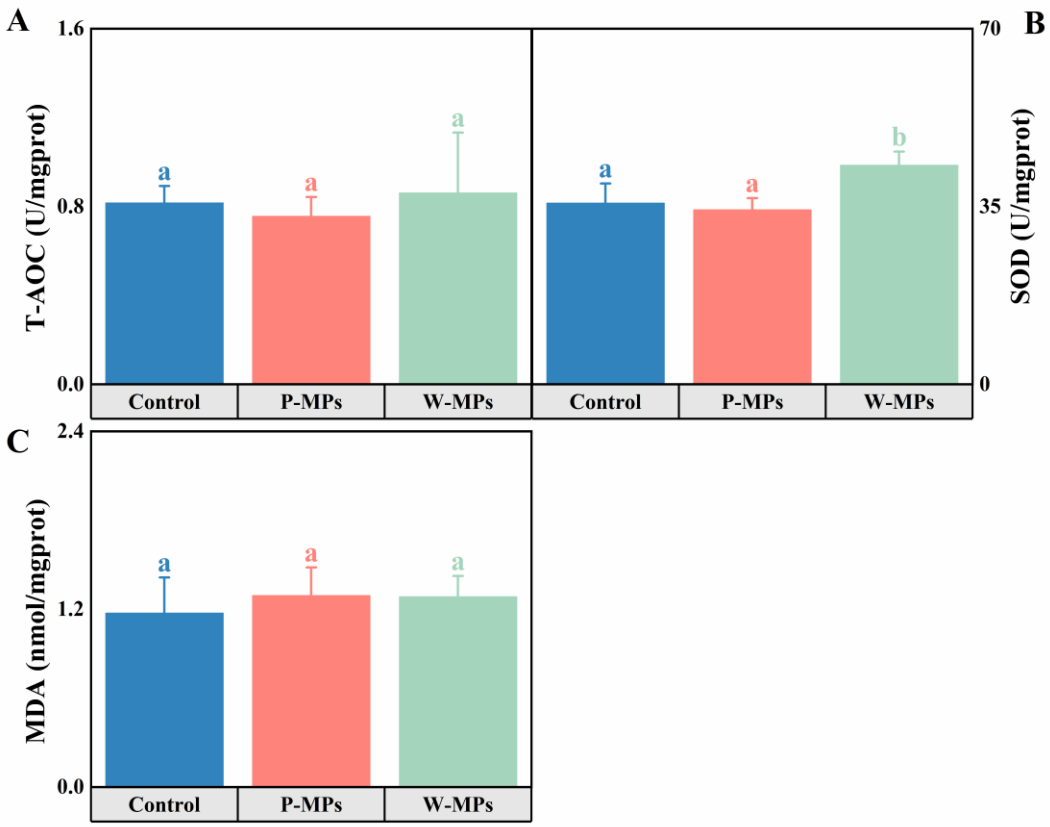


Figure 4.9. Oxidative stress in snail after exposure to pristine and weathered PS microplastics. A: Total antioxidant capacity (T-AOC) level; B: Superoxide dismutase (SOD) activity; C: Malondialdehyde (MDA) content. P-MPs represent pristine microplastics and W-MPs represent weathered microplastics. Data represent mean ± SD. Different letters indicate significant differences between treatments ($p < 0.05$).

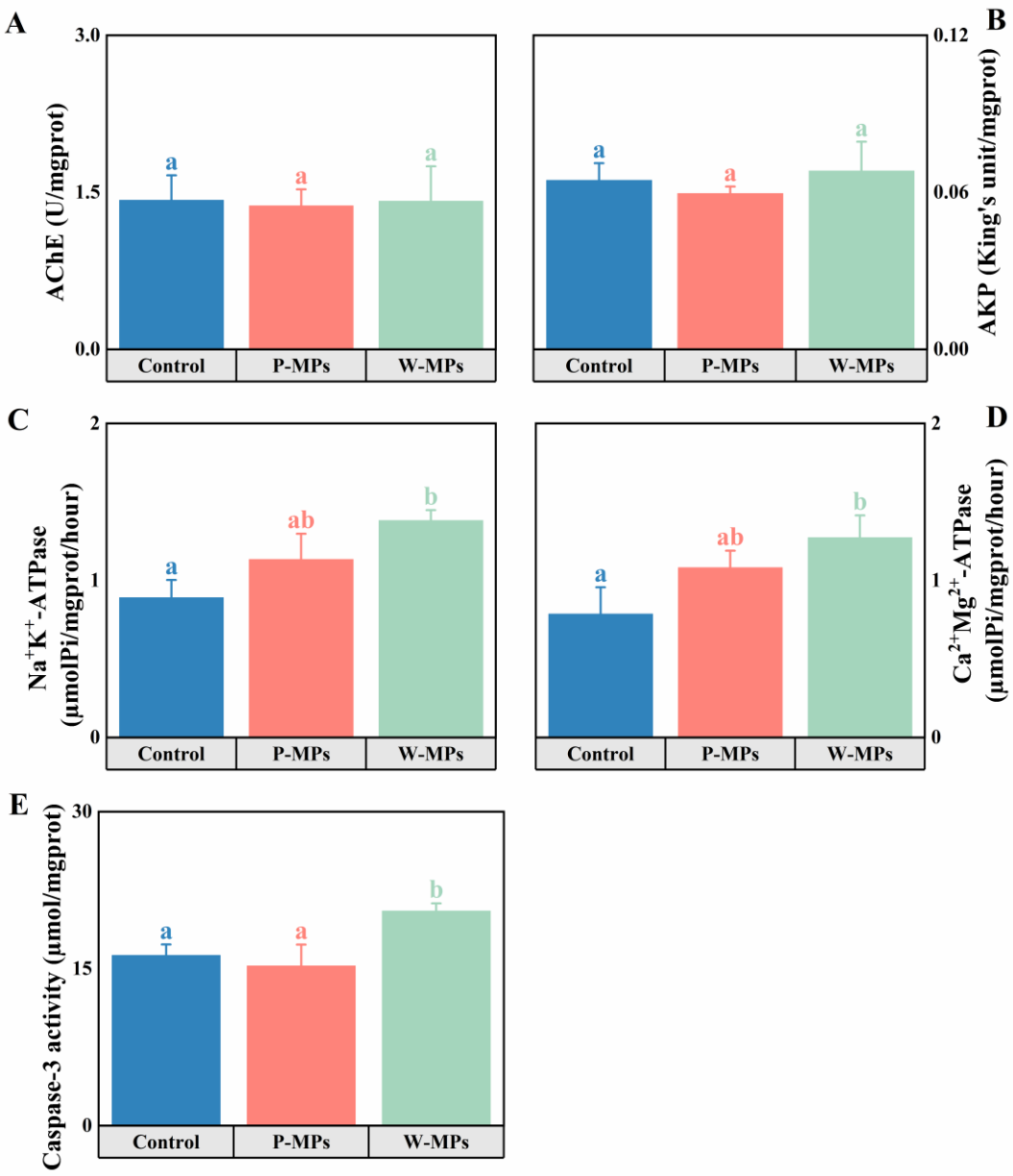


Figure 4.10. Enzyme activities of snail after exposure to pristine and weathered PS microplastics. A: Acetylcholinesterase (AChE); B: Alkaline phosphatase (AKP); C: $Na^{+}K^{+}$ATPase; D: $Ca^{2+}Mg^{2+}$ATPase; E: Caspase-3. P-MPs represent pristine microplastics and W-MPs represent weathered microplastics. Data represent mean ± SD. Different letters indicate significant differences between treatments ($p < 0.05$).

Biological responses in V. spiralis and L. stagnalis revealed adverse impacts induced by microplastics. In V. spiralis, exposure to weathered PS microplastics significantly reduced Chl-a and carotenoid levels (Fig. 4.4), indicating impaired photosynthetic efficiency. This result is in alignment with several studies which have demonstrated that

microplastics can impair photosynthetic pigment content and impact photosynthetic efficiency through physical adherence and physiological interference (Chen et al., 2025; Yu et al., 2022b; Yu et al., 2020). Microplastics can adhere to plant surfaces via electrostatic interaction, hydrophobic interaction, and surface roughness, leading to a shadowing effect that reduces the light-receiving area and, consequently, photosynthesis (Chen et al., 2025). The SEM results in our study confirmed such adhesion, particularly in the weathered microplastic group (Fig. 4.5 and 4.6). Increased SOD and MDA levels in the weathered PS microplastic group confirmed oxidative stress and membrane lipid peroxidation (Fig. 4.7). Hu et al. (2024) also found that UV-weathered polyvinyl chloride (PVC) microplastics induced more adverse impacts on the submerged aquatic species, Vallisneria natans, than pristine PVC. Elevated soluble sugars and amino acids, together with decreased levels of starch, was suggested to show metabolic reallocation under stress (Fig. 4.8). The increased soluble sugar level in our study may be a stress resistance mechanism to assist in eliminating ROS and an antiphytotoxic mechanism that compensates for reduced protein content, which was also found in a previous study (Guo et al., 2022). As the main product of carbon metabolism, the decrease of starch level in the weathered microplastic group indicated impaired carbon fixation and altered carbohydrate metabolism (Yang et al., 2021b). Decreased starch level is regarded as a stress tolerance mechanism in plants, which can enhance hydrolysis and reduce synthesis, leading to the breakdown of starch and proteins into small molecules like amino acids or soluble sugars and the release of energy (Qiu et al., 2025; Zhang et al., 2022b). Consistent with these findings, our results imply that weathered PS microplastics triggered plant defence responses and interfered with carbohydrate metabolism, energy balance, and carbon fixation to a greater extent than pristine microplastics.

In *L. stagnalis*, weathered PS microplastic exposure increased SOD, caspase-3, and ATPase activities, while AChE and AKP remained unchanged (Fig. 4.9 and 4.10). Elevated SOD activity indicated activation of antioxidant defences against ROS, reflecting oxidative stress induced by weathered PS exposure (Abdel-Tawab et al., 2021). The increase in caspase-3 suggested that oxidative damage might trigger apoptosis, consistent with previous observations in freshwater snails exposed to nanoparticles

(Abdel-Tawab et al., 2021; Florentin & Arama, 2012; Gnatyshyna et al., 2020). Programmed cell death may also be a result of oxidative stress and part of immune defence mechanisms of gastropods against pathogenic or environmental stressors (Bergmann et al., 2011). Increased Na^+/K^+-ATPase activity likely reflected compensatory ion transport to restore gradients disrupted by oxidative membrane injury (Copatti et al., 2019; Oluah et al., 2020). Similar responses have been reported in zebrafish exposed to polyethylene microplastics, where ATPase activity was enhanced to maintain ionic balance and mitigate lipid peroxidation (Rangasamy et al., 2022). Collectively, these enzyme responses demonstrate that weathered PS microplastics impose oxidative and energetic burdens on *L. stagnalis*, with apoptosis and ion-regulation pathways acting as compensatory defence mechanisms. Although no mortality occurred, these changes reveal physiological strain.

The IBR analysis quantitatively supported these results by summarizing multiple biochemical endpoints into a single stress index (Beliaeff & Burgeot, 2002; Devin et al., 2014). Compared to the pristine PS microplastic group, higher IBR scores under weathered PS microplastic exposure reflected that weathered microplastics exert greater biological stress on *V. spiralis* and *L. stagnalis* in the microcosm (Fig. 4.11).

4.4.4 Environmental multifunctionality (EMF) as an integrative indicator of microplastic effects

The EMF of the freshwater-sediment microcosm was significantly higher in both pristine and weathered PS microplastic treatments compared to the control (Fig. 4.11C). Carbon-related sediment enzymes and microbial respiration were the dominant contributors to EMF.

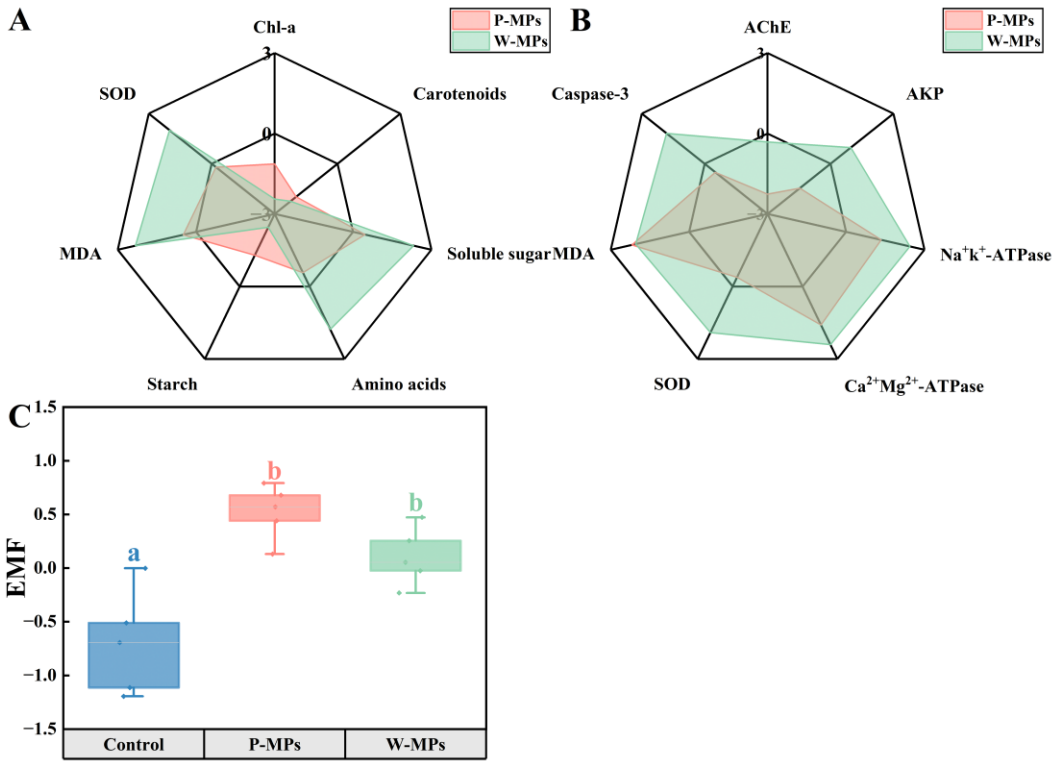


Figure 4.11. Integrated biomarker response (IBR) analysis and environmental multifunctionality (EMF) of freshwater-sediment microcosm after exposure to pristine and weathered polystyrene (PS) microplastics. A: IBR analysis of plant *V. spiralis*; B: IBR analysis of snail *L. stagnalis*; C: EMF of freshwater-sediment microcosm. P-MPs represent pristine microplastics and W-MPs represent weathered microplastics.

EMF is used to evaluate ecosystem functions and services under environmental stress and pollution, integrating various biogeochemical pathways, such as nutrient cycling, organic matter decomposition and productivity (Liu et al., 2024b). Despite its growing use in terrestrial systems, its application to microplastic-contaminated freshwater sediments remains limited (Delgado-Baquerizo et al., 2016).

In our study, both pristine and mechanically weathered PS microplastics significantly increased EMF despite inducing stress-related responses in *V. spiralis* and *L. stagnalis*, including reduced photosynthetic pigments, oxidative stress, and apoptosis (Fig. 4.12). This divergence indicates that sediment-related functional performance does not necessarily decline in parallel with organism-level stress responses. The increased EMF in our study aligns with previous research demonstrating that a relatively low concentration of PS nanoplastics in soils (0.3 g/kg) had a positive impact on EMF (Zhou

et al., 2023). Liu et al. (2024a) reported 1% PE microplastics significantly improved soil EMF by influencing the activity of α/β-1, 4-glucosidase, urease and protease, and ammonium-N content. Such similar increases in soil EMF under microplastic exposure have been linked to stimulated microbial activity and altered nutrient cycling.

Mechanistically, EMF enhancement in our microcosm was primarily driven by carbon-related processes, including elevated BG activity, increased microbial respiration, and higher DOC concentrations, which reflected intensified microbial carbon cycling and greater substrate turnover. Microplastic-induced stimulation of soil respiration and carbon flux has been reported in several studies (Gao et al., 2021; Wang et al., 2024), suggesting that altered sediment physicochemical properties and additional carbon inputs can promote microbial metabolic activity. Thus, microplastic-induced increases in sediment carbon availability and physicochemical alteration may stimulate microbial processes that temporarily sustain ecosystem output. In addition, PS is generally resistant to rapid biodegradation because PS polymers have a stable carbon-carbon backbone, hydrophobic structure and aromatic phenyl groups, which limit enzymatic attack and microbial assimilation (Ho et al., 2018; Mohanan et al., 2020). Although partial microbial degradation of PS has been reported under specific experimental conditions, such degradation is usually slow, incomplete and dependent on specialised microbial communities, prior oxidation, surface modification or long exposure periods (Yang et al., 2015; Chauhan et al., 2018). Therefore, although mechanical weathering produced rougher particle surfaces and the microcosm was likely nutrient-limited, direct utilisation of the PS polymer as a supplementary carbon source is unlikely to be the main contributor for the observed EMF increase. Instead, the EMF increase in this chapter is likely related to changes in carbon availability, dissolved organic carbon dynamics and microbial processing of existing organic substrates, rather than rapid microbial degradation of the PS polymer itself.

However, accelerated microbial metabolism may also involve higher metabolic energy demand, faster enzyme turnover, and greater consumption of available carbon substrates. While this metabolic intensification may support short-term functional stability, it could potentially alter organic carbon availability, redox conditions and

microbial community composition over longer exposure periods (Folke et al., 2004; Allison et al., 2008). Thus, the observed increase in EMF may reflect short-term functional buffering that temporarily masks organismal stress at the organism level. If elevated microbial activity continues over longer exposure periods, it could potentially alter organic carbon availability, redox conditions, nutrient balance and microbial community composition. However, longer-term studies are required to determine whether the observed response represents transient functional compensation, ecosystem rebalancing, or eventual functional deterioration.

Collectively, these findings demonstrate that EMF is a valuable integrative metric for assessing ecosystem-scale functional responses to microplastic pollution. Integrating organism-level stress indicators with multifunctionality metrics is essential to distinguish short-term functional stimulation from sustained ecosystem resilience.

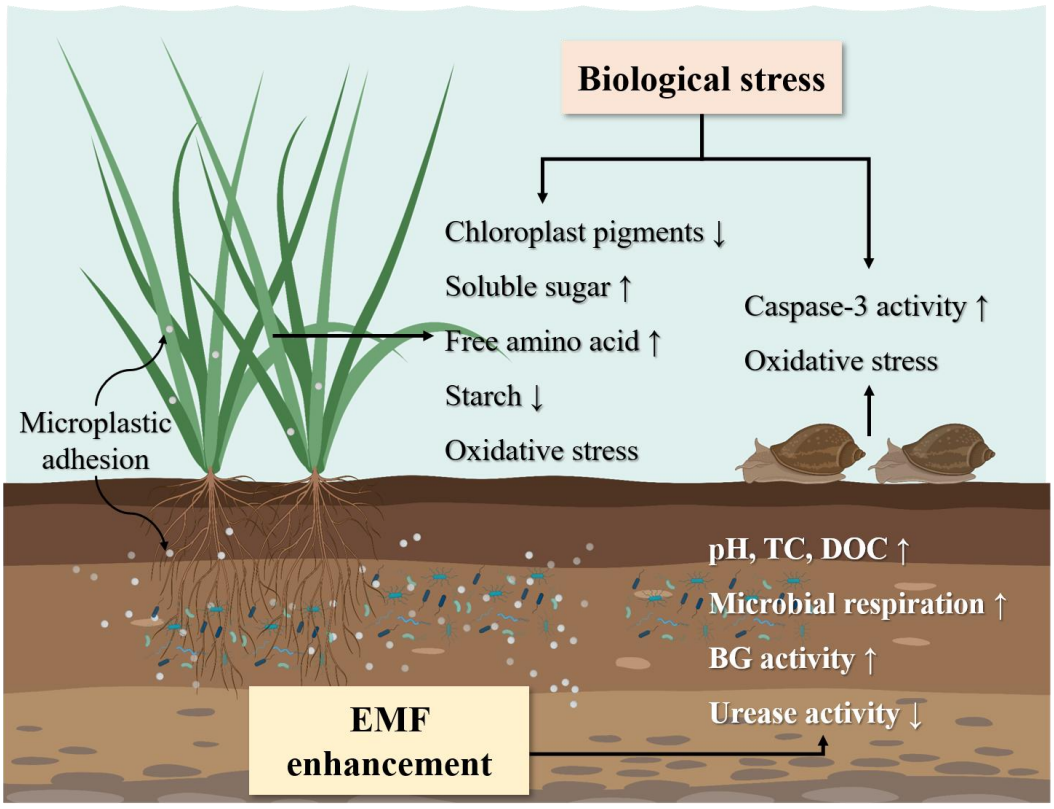


Figure 4.12. Overall effects of polystyrene (PS) microplastics on freshwater-sediment microcosm.

4.4.5 Environmental implications

This study indicated mechanically weathered PS microplastics induced intensified biological stress (high IBR value), stimulated microbial metabolism, and further enhanced EMF. Increased EMF reflected short-term stimulation of sediment carbon processing instead of improved ecosystem health. These findings emphasize the importance of integrating microplastic weathering and multifunctionality frameworks into microplastic risk assessments. Evaluations focused solely on risks to single biochemical endpoints or single species might underestimate ecological consequences (Liu et al., 2024b; Wang et al., 2024; Zhou et al., 2023). If a greater diversity of macrophytes, benthic invertebrates and microbial taxa were included, the EMF response could differ substantially from that observed in this simplified system. Higher biodiversity may increase functional redundancy and buffer sediment processes against microplastic disturbance; alternatively, additional grazers, deposit feeders or microbial decomposers may intensify particle redistribution, organic matter turnover and trophic transfer. Future studies should therefore use more complex mesocosms containing multiple macrophyte species, benthic grazers, detritivores and field-derived microbial communities, combined with nutrient-gradient and time-series designs, to determine whether the EMF enhancement observed here reflects general ecosystem buffering or a transient response of a simplified community.

4.4.6 Limitations

This chapter has several limitations. First, the experiment used artificial sediment and deionised water rather than field-collected sediment and natural freshwater. This controlled design reduced environmental variability but limited the ecological realism of the system, particularly in terms of natural dissolved organic matter, ionic composition and indigenous microbial communities. Second, no external nutrient solution was added during the five-week exposure, so the microcosms likely represented nutrient-limited conditions. The observed plant, snail and sediment microbial responses may therefore reflect the combined effects of PS microplastic exposure and nutrient limitation. Third, the experimental community was simplified, containing one submerged macrophyte, one

benthic snail species and sediment-associated microorganisms. Natural freshwater ecosystems contain more diverse producers, consumers, decomposers and trophic interactions, which could buffer or amplify microplastic effects. Fourth, the study used a single polymer type and a single exposure concentration, preventing assessment of polymer-specific or concentration-dependent responses. Fifth, biological and ecosystem-function endpoints were measured only at the end of the five-week exposure. This single time point limits interpretation of response dynamics and prevents direct inference of long-term ecological trajectories. Finally, EMF was calculated from selected sediment-associated functions and did not include organismal health indicators. The EMF increase observed in this chapter should therefore be interpreted as short-term sediment functional stimulation, not as evidence of improved ecosystem health.

4.5 Conclusions

This study demonstrated that mechanical weathering modified the surface morphology of PS microplastics and altered organismal stress responses and functional responses in a simplified freshwater-sediment microcosm. Mechanical weathering increased the surface roughness and irregularity of PS microplastics, which may have contributed to stronger interactions with *V. spiralis* and *L. stagnalis*.

Mechanically weathered PS microplastics induced stronger organismal stress responses than pristine PS microplastics, as indicated by higher IBRv2 values in *V. spiralis* and *L. stagnalis*. In *V. spiralis*, mechanically weathered PS microplastics reduced photosynthetic pigment contents and altered oxidative stress and nutrient related indicators. In *L. stagnalis*, mechanically weathered PS microplastics affected oxidative stress and enzyme activity responses, including increased SOD, ATPase and caspase-3 activities. These findings suggest that mechanical weathering can modify the biological effects of PS microplastics and intensify stress-related responses in *V. spiralis* and *L. stagnalis*. PS microplastic exposure also altered sediment-associated functional responses. Increased BG activity, sediment respiration and DOC content contributed to higher EMF under PS microplastic exposure.

Together, these findings suggest a potential tradeoff between organismal stress

responses and short-term sediment functional responses under PS microplastic exposure. Enhanced microbial carbon cycling buffered sediment functions, maintaining short-term functional stability despite biological stress. However, such stability appears to be sustained through elevated metabolic effort and accelerated energy turnover.

Overall, our study provides an integrative framework linking microplastic weathering, organismal stress (IBR), and EMF. These findings underscore the importance of incorporating microplastic weathering and multifunctionality concepts into risk assessments of microplastics in freshwater ecosystems.

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Chapter 5. Synthesis

5.1 Summary of key findings

The overarching aim of this thesis was to investigate the biological effects and potential toxicity-related mechanisms of polystyrene (PS) micro- and nanoplastics in freshwater experimental systems, while also examining their implications for sediment-associated ecosystem processes. The synthesis integrates the findings from Chapters 2-4 while recognising the important differences in experimental design among the three chapters. The thesis focused on PS particles, but the particle size class, exposure concentration, weathering process and biological level of organisation differed among chapters. Chapter 2 examined 1 μm PS microplastics in adult zebrafish (*Danio rerio*) at 30 mg/L, with particular emphasis on sex-specific sensitivity. Having identified treatment-related physiological, biochemical, reproductive and gut-liver responses in zebrafish, Chapter 3 examined 500 nm PS nanoplastics in adult zebrafish at 2.5 mg/L, comparing pristine and ultraviolet (UV)-aged particles and assessing their effects in relation to gut-liver interactions. Chapter 4 then expanded the research focus to a simplified freshwater-sediment microcosm containing the submerged macrophyte *Vallisneria spiralis*, the freshwater snail *Lymnaea stagnalis* and artificial sediment, in which pristine and mechanically weathered PS microplastics were added at 0.1% sediment dry weight.

Taken together, these chapters provide an integrated perspective on how PS micro- and nanoplastics may induce stress-related responses in adult zebrafish, *V. spiralis* and *L. stagnalis*, as well as alter sediment-associated microbial and biogeochemical processes. The findings encompass direct organismal responses, including oxidative stress, immune-related changes, gut microbiota disturbance, metabolic alterations, pigment responses and enzyme activity, together with indirect ecological responses involving sediment respiration, enzyme activity and ecosystem multifunctionality (EMF). However, the evidence generated in this thesis is strongest for stress-related and adverse biological responses under controlled experimental conditions rather than for direct, irreversible toxicity.

The three chapters cannot be compared as a direct dose-response, size-response or weathering-method comparison. Chapter 2 used 1 μm PS microplastics, whereas Chapter 3 used 500 nm PS nanoplastics, and the exposure concentrations differed substantially between these experiments. In addition, UV aging in Chapter 3 and mechanical weathering in Chapter 4 were examined in separate experimental systems. These processes therefore should not be treated as interchangeable or ranked universally in terms of hazard. Instead, the synthesis identifies recurring patterns and conceptual links across the thesis while keeping the interpretation confined to the specific particle types, exposure conditions and organisms tested. To highlight the specific contributions of this research, the key findings are discussed below in relation to each experimental chapter, with emphasis on their novelty, significance and limitations.

5.1.1 Gender-specific responses to pristine polystyrene (PS) microplastics in zebrafish (Chapter 2)

Chapter 2 provides evidence that PS microplastics induce gender-specific stress-related biological responses and reproductive changes in adult zebrafish. While previous studies have documented oxidative stress and metabolic disruption in fish exposed to microplastics, most have either pooled sexes or focused on juveniles, thereby overlooking gender-dependent responses. In contrast, the present study compared responses of male and female zebrafish and showed that females exhibited stronger oxidative stress, metabolic disturbance, and reproductive alterations (Fig. 2.2, 2.7-2.10).

By linking indicators of oxidative stress, gut microbiota, liver metabolomics, and reproductive process, Chapter 2 helps interpret how PS microplastic exposure may be associated with metabolic and reproduction-related responses in female zebrafish. These findings suggest that biological sex is an important factor influencing zebrafish responses to PS microplastic exposure. Such effects are particularly relevant at the population level, as alterations of female reproductive process can lead to long-term population decline even when male health appears relatively unaffected.

5.1.2 UV aging modifies stress-related biological responses of PS nanoplastics via gut-liver axis (Chapter 3)

A major contribution of this thesis lies in demonstrating that UV aging alters the physicochemical properties and stress-related biological responses of PS nanoplastics. Although environmental aging is widely recognized as an inevitable fate of plastics in aquatic systems, it is still underrepresented in experimental toxicology. Chapter 3 addresses this gap by linking UV-induced physicochemical changes in PS nanoplastic properties with altered biological responses in zebrafish.

Chapter 3 showed that UV-aged PS nanoplastics were associated with stronger stress-related responses than pristine PS nanoplastics under the tested conditions. These responses included changes in skin mucus indicators, hepatic oxidative stress biomarkers, gut microbiota composition and metabolic alteration through gut-liver axis (Fig. 3.3, 3.4 and 3.7). Both pristine and UV-aged PS nanoplastics were associated with gut microbiota alteration and hepatic metabolic disturbance, but the female UV-aged PS nanoplastic treatment group showed the broadest response pattern. The combined microbial, biochemical and metabolic evidence suggests that UV aging may modify how PS nanoplastics interact with zebrafish tissues and biological regulatory systems.

These findings highlight the importance of accounting for aging processes in micro- and nanoplastic risk assessment. In freshwater environments, micro- and nanoplastics rarely remain in a pristine state; instead, they undergo various weathering processes. The results of Chapter 3 demonstrate that ignoring aging processes may lead to substantial underestimation of ecological risk.

5.1.3 Scaling from organismal stress to ecosystem processes (Chapter 4)

Chapter 4 extends the scope of this thesis beyond individual-level responses by investigating how pristine and mechanically weathered PS microplastics influence a freshwater-sediment microcosm. This chapter makes a novel contribution by linking stress responses of submerged macrophyte *V. spiralis* and freshwater snail *L. stagnalis* with changes in EMF, which remains relatively underexplored in microplastic research.

Although plants and snails exhibited obvious stress-related responses (Fig. 4.4, 4.7-

4.10), EMF increased under microplastic exposure. This increase of EMF was associated with enhanced microbial respiration and enzymatic activity, indicating that carbon-related processes were stimulated. These findings reveal that ecosystem function can be maintained or even enhanced when organism-level stress responses are present.

By showing that functional stability can coexist with organismal stress, this chapter highlights the risk of relying solely on ecosystem-level indicators without considering underlying energetic and physiological costs.

The following sections synthesize the findings from the thesis and discuss their connections.

5.1.4 Stress-related responses versus direct toxicity-related adverse outcomes: what the thesis does and does not show

A key conceptual refinement in this thesis is the distinction between stress-related biological responses and direct toxicity-related adverse outcomes. Stress-related responses refer to physiological, biochemical, microbial or metabolic changes that indicate disturbance of homeostasis under exposure pressure. These responses may represent early-warning signals, compensatory adjustment or biological disturbance, and may be reversible if the stressor is removed or if the organism acclimates. However, the direction of change alone does not determine whether a response is adaptive or harmful. Interpretation therefore requires consideration of the biomarker measured, its biological function, the magnitude and duration of change, and whether the response is accompanied by impairment at a higher level of biological organisation.

As shown in Table 5.1, the oxidative-stress results in this thesis provide evidence of redox disturbance. Changes in microbial community composition, diversity or relative abundance may reflect disturbance, host adaptation, altered feeding, changes in gut transit, particle-associated microbial colonisation or shifts in available substrates. Stronger evidence of dysbiosis would require integration of taxonomic data with microbial functional genes, metabolite profiles, intestinal permeability, inflammatory markers and host fitness endpoints. Some findings may be interpreted as potential adverse endpoints. Reduced egg production in pairings involving exposed female zebrafish may indicate

altered reproductive investment, endocrine regulation or energy allocation. Increased caspase-3 activity in *L. stagnalis* indicates activation of apoptosis-related pathways. Reduced pigment content in *V. spiralis* suggests photosynthesis-related stress or possible photosynthetic impairment.

The evidence generated in this thesis is therefore strongest for stress-related physiological, microbial and metabolic responses rather than direct toxicity. No mortality was observed or reported, and clear evidence of sustained growth inhibition, biomass loss, organ damage or long-term reproductive failure was limited or absent. The increased ecosystem multifunctionality observed in the sediment microcosm, driven by DOC, β-glucosidase and respiration, should be interpreted as a short-term functional response rather than evidence of improved ecosystem health or resilience.

Overall, PS micro- and nanoplastics induced oxidative, immune-related, microbial, metabolic, reproduction-related, photosynthesis-related and sediment-associated responses in the tested systems. However, these findings do not demonstrate direct lethality, irreversible organismal damage or long-term population decline. This distinction provides an important interpretive boundary for the synthesis: the thesis demonstrates sublethal stress-related responses and potential toxicity-related effects under controlled experimental conditions, but further long-term, recovery and fitness-based studies are required to determine whether these responses translate into persistent adverse outcomes.

5.2 UV aging and mechanical weathering alter microplastic properties, intensifying biological responses: Similarities, differences and implications

UV aging and mechanical weathering represent distinct transformation pathways and should not be treated as equivalent aging processes. In Chapter 3, UV aging was applied to 500 nm PS nanoplastics and primarily induced photooxidative changes in particle surface chemistry and morphology. These changes may include polymer-chain scission, the formation of oxygen-containing functional groups such as carbonyl, hydroxyl and carboxyl groups, increased surface oxidation and the development of rougher surfaces. In contrast, Chapter 4 examined mechanically weathered PS microplastics, representing physical abrasion and fragmentation. Mechanical weathering mainly produced rougher

and more irregular particles. These physical changes could increase particle aggregation, adhesion to biological tissues and interactions with sediment minerals, organic matter and microorganisms.

Table 5.1. Summary of evidence categories used to distinguish stress-related responses, potential toxicity-related endpoints and ecosystem-level functional responses in this thesis.

Evidence category	Supported by this thesis	Interpretation
Stress-related responses	Superoxide dismutase (SOD), malondialdehyde (MDA), reactive oxygen species (ROS), immunoglobulin M (IgM), lipopolysaccharide (LPS), triglyceride (TG) changes in zebrafish; microbiota and metabolomic shifts; plant SOD/MDA and pigment changes; snail SOD, ATPase; sediment β-glucosidase and respiration	Stress-related physiological, microbial and metabolic responses
Potential toxicity-related endpoints	Reduced egg production in pairings involving exposed female zebrafish; possible plant photosynthetic impairment; apoptosis-related enzyme response in snails	Potential adverse outcomes; require support from persistence, functional impairment or recovery data
Direct toxicity not demonstrated	Mortality, growth inhibition, biomass loss, long-term reproductive failure	Not supported or not measured
Sediment-related functional response	Increased ecosystem multifunctionality (EMF) driven by dissolved organic carbon (DOC), β-glucosidase and respiration	Short-term functional stimulation

Despite their different mechanisms, UV aging and mechanical weathering share several potential consequences. Both processes can modify particle morphology, increase surface roughness and change the interaction of PS particles with biological surfaces and environmental matrices. These shared characteristics may help explain why UV-aged PS

nanoplastics in adult zebrafish and mechanically weathered PS microplastics in the freshwater-sediment microcosm were associated with stronger stress-related responses than their respective pristine treatments. In Chapter 3, UV-aged PS nanoplastics produced more pronounced oxidative, gut microbiota and gut-liver metabolic disturbances in adult zebrafish than pristine particles. In Chapter 4, mechanically weathered PS microplastics were associated with increased oxidative stress in *V. spiralis* and *L. stagnalis*, as well as changes in sediment-associated microbial processes, including β-glucosidase activity and sediment respiration. These findings indicate that particle transformation can influence biological and ecosystem-associated responses, but they do not demonstrate that UV aging and mechanical weathering have equivalent effects. These findings are consistent with studies showing that weathering processes can modify the surface properties, environmental behaviour and biological interactions of micro- and nanoplastics, thereby altering their ecotoxicological effects under specific exposure conditions (Gewert et al., 2015; Li et al., 2022a; Michailidou et al., 2024).

The relative importance of each weathering pathway is likely to depend on environmental context. UV aging may be particularly relevant for particles exposed in surface waters, shallow water columns or shoreline environments, where solar radiation can promote photooxidation. Mechanical weathering may be more important in sediments, resuspension zones and environments characterised by frequent particle and mineral contact, water movement or abrasion. In natural freshwater environments, microplastics seldom remain pristine for long. Experimental and field studies indicate that physicochemical changes can occur within weeks to months of environmental exposure, particularly under ultraviolet radiation and mechanical stress (Gewert et al., 2015; Lambert & Wagner, 2016). In addition, factors such as fluctuating temperatures, mechanical abrasion, microbial colonization, and interactions with organic matter can alter their properties (Liu et al., 2019; Rummel et al., 2017).

Accordingly, the findings of this thesis support the inclusion of environmentally transformed PS particles in ecotoxicological assessments, but they do not justify the general conclusion that all aged particles are more toxic than pristine particles. Rather, the results suggest that specific weathering processes can modify particle properties and

biological interactions under particular experimental conditions. Future studies should therefore examine UV aging and mechanical weathering both separately and in combination, using sequential or multi-stage treatments that better represent environmental transformation pathways. At the same time, controlled single-process experiments remain necessary to distinguish the mechanisms associated with photooxidation, physical abrasion and fragmentation. Such designs would help determine whether combined weathering produces additive, synergistic or antagonistic effects on adult zebrafish, freshwater invertebrates, aquatic plants and sediment-associated microbial processes.

5.3 Energy reallocation as a possible cross-scale framework

Across Chapters 2 and 3 of this thesis, zebrafish exposed to either pristine or UV-aged PS particles showed stress-related responses that may be associated with altered energy allocation. Indicators such as ROS, superoxide dismutase (SOD), and malondialdehyde (MDA) provided evidence of oxidative imbalance (Fig. 2.2 and 3.3). At the cellular level, oxidative stress imposes a significant energetic burden. Excess ROS can initiate lipid peroxidation and damage membranes, proteins, and other macromolecules, ultimately triggering cellular defence and repair pathways (Su et al., 2019). Increased SOD activities reflect that zebrafish attempt to clear superoxide radicals and hydrogen peroxide, while increased MDA content indicates lipid peroxidation and membrane damage (Gulcin, 2025; Wen et al., 2025). Therefore, these biochemical responses suggest that PS particle exposure may increase the energetic demand required to maintain cellular function under stress. (Abele & Puntarulo, 2004; Lushchak, 2011).

As seen in Chapter 2 (section 2.4.5), reproduction-related changes, including altered sex hormone levels and reduced egg production in pairings involving exposed females, were observed together with oxidative-stress responses, gut microbiota disturbance and liver metabolic alteration. These results are consistent with a growing body of literature showing that microplastics can impair reproductive performance, disrupt lipid metabolism, and reduce body condition in fish and other aquatic organisms (Lu et al., 2016; Wang et al., 2019a; Wang et al., 2019b). The freshwater-sediment microcosm

experiment (Chapter 4) revealed a similar pattern at the ecosystem level. Microbial communities responded to microplastic-induced disturbance by increasing carbon metabolism, as reflected in elevated respiration rates and enzymatic activities (Fig. 4.3). At the same time, processes associated with nitrogen cycling, such as urease activity, declined (Fig. 4.3). These changes reveal that carbon processing was enhanced while nitrogen transformations were suppressed. Despite the stress observed in plants and snails, these microbial adjustments contributed to the maintenance of sediment-related ecosystem functioning. In conclusion, these findings suggest that sediment microbial communities may have shifted their activity toward decomposition, nutrient cycling and carbon transformation in response to microplastic exposure.

Increased β-glucosidase activity indicates enhanced carbohydrate breakdown and carbon cycling, which are likely to require additional metabolic activity and energy. Elevated microbial respiration further reflects the increase in oxidative metabolism, suggesting that microbial communities may increase energy use to maintain nutrient turnover in the presence of microplastics. These responses align with several studies showing that microplastic exposure triggers microbial community shifts toward faster-growing or more stress-tolerant taxa with higher metabolic rates (Chomiak et al., 2024; Li et al., 2022b; Tao et al., 2024; Zhang et al., 2023). Thus, microbial communities may respond to microplastic pollution through functional adjustment, increasing metabolic activity to support short-term sediment functional stability under stress.

This pattern is consistent with widely recognized energy-based frameworks in ecotoxicology, such as the dynamic energy budget model, which shows that organisms under pollutant exposure divert energy from fitness-related functions to survival and homeostasis (Kooijman, 2009). In this thesis, oxidative-stress responses in zebrafish and increased sediment microbial respiration suggest that PS micro- and nanoplastic exposure may alter energy-related processes at both organismal and sediment-associated functional levels. Such changes may support short-term physiological adjustment in organisms or short-term functional stimulation in sediments. However, biological stress in individuals may affect reproduction-related processes, while persistent stimulation of microbial activity could potentially alter nutrient balance, carbon turnover and sediment

biogeochemical processes over longer time scales. Therefore, energy adjustment may help explain short-term response patterns, but its longer-term ecological consequences require further validation.

In summary, the results of this thesis suggest that energy-related responses provide a useful interpretive framework for linking PS micro- and nanoplastic effects across biological scales. From oxidative-stress responses in zebrafish to changes in sediment microbial activity, altered energy demand and resource allocation may help explain how PS particles influence freshwater organisms and sediment-associated processes. This work highlights the importance of considering both immediate stress-related responses and possible longer-term ecological consequences when assessing micro- and nanoplastic pollution.

5.4 Gender-specific sensitivity: from individual response to population implications

In both pristine PS microplastic exposure in Chapter 2 and UV-aged PS nanoplastic exposure in Chapter 3, adult female zebrafish showed broader biochemical and physiological stress responses than males, including oxidative stress, gut microbiota disturbance, hepatic or gut-liver metabolic alterations, and reproduction-related changes. These findings suggest that biological sex is an important source of variability in zebrafish responses to PS particle exposure. Although the thesis did not explore the underlying mechanisms, previous research indicates that the gender-biased responses may reflect differences in energy allocation, hormonal regulation, or antioxidant physiology (Niksirat et al., 2025; Zhu et al., 2023).

Several mechanisms may explain why female zebrafish showed stronger responses in this thesis. First, adult female zebrafish have high reproductive energy demands associated with oocyte development, vitellogenesis, steroid hormone regulation and repeated spawning. In fish, vitellogenesis is regulated by oestradiol and involves hepatic synthesis of vitellogenin, which is transported to developing oocytes and provides yolk nutrients for embryonic development (Levi et al., 2009; Zheng et al., 2013). Therefore, when PS microplastic and nanoplastic exposure induces oxidative stress or metabolic disturbance, females may experience a stronger trade-off between maintaining

reproductive investment and allocating energy to antioxidant defence, cellular repair and detoxification. Previous study also shows that stress can affect reproduction through energy re-budgeting and allostatic responses (Schreck, 2010).

Second, hepatic metabolism is likely to be related to the female-biased response. The zebrafish liver is sexually dimorphic at the transcriptomic level, and sex hormones regulate hepatic gene expression related to lipid metabolism, reproduction and xenobiotic metabolism (Zheng et al., 2013). More recent proteomic evidence also indicates that female zebrafish liver is strongly associated with anabolic processes supporting vitellogenesis and egg production, whereas male liver shows a different metabolic and detoxification profile (Niksirat et al., 2025). Therefore, disruption of hepatic lipid, amino acid or carbohydrate metabolism may have stronger reproductive consequences in females than in males. This provides a plausible explanation for why female zebrafish in Chapters 2 and 3 showed more pronounced liver or gut-liver metabolic responses after PS particle exposure.

Third, endocrine regulation may contribute to the observed sex-specific pattern. Sex steroid hormones such as oestradiol, testosterone and 11-ketotestosterone regulate reproductive development, vitellogenesis, gonadal maturation and hepatic metabolism in fish. Studies on PS microplastics and nanoplastic exposure in zebrafish have reported changes in sex hormones, steroidogenesis-related genes, HPG/HPGL-axis genes, reproductive output and offspring outcomes, supporting the relevance of endocrine-related endpoints in future sex-specific studies (Gupta et al., 2023; Li et al., 2024). However, the endocrine mechanism in this thesis remains inferential because gene expression in the HPGL axis, gonad histology, vitellogenin and offspring viability were not directly measured.

Fourth, gut microbiota disturbance may interact with liver metabolism and endocrine responses. The gut and liver communicate through the portal circulation, bile acids, microbial metabolites, immune signals and gut-derived inflammatory molecules; intestinal factors can regulate hepatic glucose and lipid metabolism, while liver-derived products can influence gut barrier integrity and microbiota composition (Tripathi et al., 2018). In fish, PS microplastic exposure has been shown to alter gut microbiota and

metabolomic profiles in zebrafish, with changes associated with oxidative stress, inflammation and lipid metabolism (Qiao et al., 2019). Similar associations between gut microbiota and hepatic metabolism have also been reported in fish exposed to PS microplastics, supporting the interpretation that gut microbial disturbance may be linked to liver metabolic stress (Feng et al., 2021). Therefore, the stronger female gut-liver response observed in this thesis may reflect interactions among microbiota disturbance, hepatic lipid metabolism, immune signalling and endocrine regulation, although causality cannot be established from correlative omics data alone.

At the same time, it is important to acknowledge that gender-specific sensitivity to pollution is context dependent. In some cases, males have been reported to exhibit greater vulnerability to environmental contaminants, depending on pollutant type, metabolic levels between female and male resulting in different responses to environmental interventions, and differences in nutrient and energy demand or utilization between females and males (El Kholy & Al Naggar, 2023; Li et al., 2024; Teng et al., 2025). Gender-specific responses can reflect fundamental physiological differences that influence how organisms respond to environmental stressors. Accounting for this variability is therefore essential for accurately assessing the ecological risks of microplastic contamination in freshwater ecosystems.

5.5 Ecosystem stability under stress

The freshwater-sediment microcosm experiment showed that exposure to mechanically weathered PS microplastics induced measurable stress-related responses in plants and snails, affecting physiological indicators such as photosynthetic pigment contents, oxidative-stress biomarkers and nutrient-related indicators. These findings are broadly consistent with the organism-level oxidative-stress responses observed in zebrafish. The convergence of responses among fish, snails and plants suggests that PS microplastic exposure may induce recurring stress-related patterns across different freshwater organisms, particularly involving oxidative stress, altered energy-related processes and physiological regulation.

Despite the evidence of biological stress across submerged macrophyte and

freshwater snail, the freshwater-sediment microcosm still showed an increase in EMF. The maintenance of ecosystem function does not indicate an absence of disturbance; but instead highlights the capacity of microbial communities to compensate by increasing metabolic activity when submerged macrophyte and freshwater snail experience reduced physiological performance. However, it is important to highlight that this comes with an energetic cost. Sustained high microbial activity requires more metabolic energy, faster enzyme turnover, and greater use of available substrates. Over longer periods, such effort can deplete organic carbon storage, alter redox conditions, and gradually shift the composition and stability of microbial communities. Similar patterns have been reported in ecosystems exposed to other pollutants, where microbial assemblages temporarily maintain critical biogeochemical processes by operating at higher metabolic intensity until their energy reserves, substrate availability, or community diversity begin to decline (Allison & Martiny, 2008; Folke et al., 2004).

This pattern may be conceptually linked to the energy-related responses observed in zebrafish in Chapters 2 and 3. In zebrafish, oxidative-stress responses and metabolic alterations suggest that PS particle exposure may increase the energetic demand required for cellular maintenance and physiological adjustment. In the freshwater-sediment microcosm, increased β-glucosidase activity and respiration suggest stimulation of sediment carbon-processing activity. In both cases, biological systems may respond to disturbance by adjusting energy-related processes to support short-term homeostasis or functional output. However, because direct energy budgets, microbial community composition and long-term recovery were not measured, this interpretation should be considered a plausible explanatory framework rather than a demonstrated mechanism.

By highlighting the coexistence of organismal stress responses and increased sediment-associated multifunctionality, this thesis emphasises the need for a more integrated approach to ecological risk assessment. EMF is a valuable indicator for summarising multiple functional responses, but it should be evaluated together with organism-level physiological and biochemical indicators to distinguish short-term functional stimulation from sustained ecological stability or recovery.

5.6 Limitations of the present study

While this thesis provides new insights into biological responses to PS micro- and nanoplastics in freshwater experimental systems, several limitations should be acknowledged. First, the exposure scenarios used in the Chapter 2-4 differed substantially in particle size, concentration, weathering process, exposure matrix and biological level of organisation. Chapter 2 examined 1 μm PS microplastics in adult zebrafish at 30 mg/L, Chapter 3 examined 500 nm PS nanoplastics in adult zebrafish at 2.5 mg/L, and Chapter 4 examined PS microplastics added to artificial sediment at 0.1% sediment dry weight in a simplified freshwater-sediment microcosm. These exposure levels, particularly the 30 mg/L waterborne exposure used in Chapter 2, were higher than most reported environmental concentrations in freshwater systems. Therefore, they were chosen as controlled laboratory stress-test conditions designed to detect sensitive biological responses and explore potential mechanisms, rather than as direct simulations of typical field exposure. In addition, UV aging in Chapter 3 and mechanical weathering in Chapter 4 were applied in separate, non-equivalent experiments rather than tested side by side. These exposure conditions were designed to examine biological and sediment-associated responses under controlled laboratory conditions rather than direct field-level risk predictions.

Second, the study focused on a single polymer type (PS) and a limited range of particle sizes and particle forms. While PS microplastics are environmentally relevant and widely used in microplastic research, other polymers with different densities, additives, and degradation pathways may elicit distinct biological responses. Irregular fragments, fibres, films, foams and environmentally collected particles may interact with organisms and sediment differently from the controlled PS particles used here. As such, caution is warranted when extrapolating these findings to all forms of microplastic pollution. Moreover, while the present study used laboratory synthesized microplastics, UV radiation and mechanical fragmentation to simulate the weathering process, further studies could utilize more realistic microplastics or weathering methods to better simulate the weathering of microplastics in real water environments.

Third, Chapter 2-4 relied on terminal sampling at a single time point. This limits the

ability to determine whether the observed responses were transient, adaptive, progressive or reversible. In Chapters 2 and 3, gut microbiota and metabolomic changes were interpreted as evidence of physiological disturbance, but causal gut-liver mechanisms could not be confirmed without time-series data, targeted validation or intervention experiments. In Chapter 4, increased sediment respiration, β-glucosidase activity and ecosystem multifunctionality were measured only at the end of the exposure period. This single snapshot cannot determine whether the enhanced sediment-associated function represented short-term stimulation, acclimation, resilience or an early stage of longer-term deterioration.

Fourth, the gender-specific responses observed in adult zebrafish require further mechanistic validation. Female zebrafish showed broader treatment-related responses than males in Chapters 2 and 3, but the underlying mechanisms were inferred rather than directly tested. Potential mechanisms include reproductive energy demand, endocrine regulation, hepatic lipid metabolism, antioxidant capacity, immune regulation, gut microbiota differences and gender-specific internal particle burden. However, this thesis did not directly measure gonad histology, vitellogenin, sex steroid synthesis pathways, hypothalamic-pituitary-gonadal-liver axis gene expression, fertilisation success, offspring viability or recovery after exposure.

Finally, Chapter 4 used a simplified artificial freshwater-sediment microcosm. The use of artificial sediment and deionised water reduced environmental variability, but also limited ecological realism. The system lacked natural dissolved organic matter, natural ionic composition, field-derived sediment structure, established indigenous microbial communities and the trophic complexity of natural freshwater ecosystems. No external nutrients were supplied during the exposure, meaning that the system may have represented nutrient-limited conditions. The experimental community contained only one submerged macrophyte, one freshwater snail species and sediment-associated microbial processes. The interactions among microcosm components, particularly how microplastic exposure influences organism-microbe and plant-animal interactions still need further study.

5.7 Future perspectives and broader implications

The findings of this thesis have implications beyond the specific laboratory systems studied. Plastic pollution is not only a toxicological issue but also an environmental management, biodiversity and societal challenge. Freshwater ecosystems are important pathways connecting land-based plastic use, wastewater discharge, river transport, sediment accumulation and downstream marine pollution. Therefore, understanding how plastic particles behave and affect freshwater systems is relevant to water quality protection, biodiversity conservation, fisheries, recreation, and the broader societal goal of reducing plastic leakage into the environment.

First, the effects of microplastics depend on polymer type, size, shape, surface properties, additives, weathering state and environmental context. This thesis focused on PS particles, but even within this single polymer type, the responses differed according to particle size, UV aging, mechanical weathering and exposure system. This supports the wider argument that microplastics should be understood as a diverse contaminant suite rather than one simple pollutant category. For environmental monitoring and risk assessment, this means that counting particles alone is insufficient. Information on particle size, polymer type, morphology and weathering state is needed to understand potential biological relevance.

Second, weathering of microplastics deserve greater attention in environmental assessment. In real freshwater systems, microplastics are unlikely to remain pristine. They may be modified by sunlight, abrasion, sediment contact, chemical oxidation and biofilm colonisation. In this thesis, UV-aged PS nanoplastics in adult zebrafish and mechanically weathered PS microplastics in the sediment microcosm were associated with stronger stress-related responses than their pristine ones. Therefore, environmental transformation can change how plastic particles interact with organisms, sediments and microbial communities, and should therefore be included in future testing frameworks.

Third, the gender-specific responses observed in adult zebrafish show that males and females should not automatically be treated as interchangeable test organisms. If only mixed-sex or pooled samples are used, important biological differences may be masked. This matters not only for ecotoxicology but also for how environmental risk is interpreted.

A contaminant that causes moderate average effects may still place a greater burden on one sex, life stage or physiological state. For society and environmental management, this reinforces the need to consider biological diversity when assessing pollutant impacts, rather than relying only on simplified average responses.

Fourth, ecosystem-level indicators need careful interpretation. In Chapter 4, sediment-associated ecosystem multifunctionality increased under PS microplastic exposure, while *V. spiralis* and *L. stagnalis* showed stress-related responses. This suggests that ecosystem functions may appear stable or even enhanced while biological stress is present in particular organisms. For environmental monitoring, this is important because relying on one type of indicator may produce an incomplete picture. Chemical measurements, organismal biomarkers, biodiversity indicators and ecosystem-function metrics should be interpreted together to distinguish genuine ecological stability from short-term functional compensation.

Finally, the results of this thesis support concern about PS micro- and nanoplastics because they induced stress-related responses in adult zebrafish, *V. spiralis*, *L. stagnalis* and sediment-associated processes under controlled conditions. At the same time, the findings should not be overstated as proof of universal toxicity, direct lethality or long-term ecosystem collapse. For policy, regulation and public communication, this balance is important. The societal response to plastic pollution should prioritise reducing unnecessary plastic use, preventing environmental leakage, improving waste and wastewater management, and developing monitoring methods that capture the diversity and transformation of plastic particles.

5.8 Future research priorities and remaining knowledge gaps

Building on the findings and limitations of this thesis, several specific research priorities should be addressed in future work.

First, future studies should use exposure designs that better connect laboratory hazard testing with environmental risk assessment. This should include wider concentration gradients, environmentally measured concentrations, and reporting exposure in multiple units, including mass concentration, particle number, particle size distribution and, where

relevant, surface area. For sediment studies, concentrations should be reported as mass per dry sediment mass and, where possible, particle number per gram of sediment. This would improve comparability among studies and help determine whether stress-related responses observed under controlled laboratory conditions occur at environmentally realistic exposure levels.

Second, future work should compare different particle transformation pathways in a more systematic way. UV aging, mechanical weathering, chemical oxidation, biofilm colonisation and natural field weathering should be tested both separately and in sequential or combined designs. For example, future experiments could compare pristine PS particles, UV-aged particles, mechanically weathered particles, biofilm-colonised particles and particles subjected to combined UV exposure followed by mechanical abrasion. Particle characterisation should include SEM or TEM imaging, particle-size distribution, zeta potential, hydrodynamic size, FTIR, XPS, carbonyl index, O/C ratio and leachate analysis. The implication of this work would be to determine whether weathering effects are driven mainly by surface chemistry, roughness, fragmentation, particle size, biofilm formation or additive release.

Third, sex-specific mechanisms in adult zebrafish should be investigated directly. Future studies should include sex as an explicit experimental factor and test sex, treatment and sex × treatment interactions statistically. Measurements should include internal particle burden, uptake and depuration kinetics, feeding rate, gut transit time, gonadosomatic index, gonad histology, oocyte stage, vitellogenin, oestradiol, testosterone, 11-ketotestosterone, fertilisation success, embryo viability and offspring development. Molecular endpoints should include genes related to steroidogenesis, oestrogen and androgen signalling, oxidative stress, detoxification, inflammation and the hypothalamic-pituitary-gonadal-liver axis. The implication would be to determine whether female-biased responses are driven by reproductive energy demand, endocrine disruption, hepatic metabolism, gut-liver interactions, internal exposure differences or combined mechanisms (Zheng et al., 2013; Gupta et al., 2023).

Fourth, future studies should distinguish adaptive stress responses from direct toxicity more clearly. Biomarkers such as ROS, SOD, CAT, GPx, MDA, immune

indicators, microbiota composition and metabolomic profiles are valuable early-warning indicators, but they should be linked with apical and fitness-related endpoints. Future work should therefore include survival, growth, body condition, biomass, histology, reproduction, behaviour, feeding performance and recovery after exposure. Recovery experiments are particularly important because they can determine whether biomarker changes are reversible acclimation responses or signs of persistent damage. The implication would be to strengthen the ecological interpretation of sublethal biomarker responses and avoid overclaiming toxicity from molecular or biochemical endpoints alone.

Fifth, the gut-liver axis mechanism should be tested using targeted and causal approaches. In zebrafish, future experiments should combine microbiome sequencing with targeted metabolomics, bile acid profiling, lipidomic, gut barrier markers, inflammatory cytokines and hepatic detoxification markers. Time-series sampling would help determine whether gut microbiota shifts precede liver metabolic changes, or whether both occur as parallel responses to particle exposure. Microbiota manipulation, probiotic or antibiotic treatments, germ-free models, or faecal microbiota transfer could be used to test causality more directly. The implication would be to move from associative omics patterns toward mechanistic understanding of how PS particles influence host metabolism and immune regulation through gut-liver communication (Qiao et al., 2019; Tripathi et al., 2018).

Sixth, freshwater-sediment microcosm studies should become more environmentally realistic and mechanistically informative. Future systems should compare artificial sediment with field-collected sediment, deionised water with natural freshwater, and nutrient-limited with nutrient-replete conditions. They should also include more complex biological communities, such as multiple submerged macrophyte species, grazers, detritivores, filter feeders and field-derived microbial communities. Key measurements should include plant and invertebrate growth, biomass, reproduction, survival, microbial community composition, functional genes, DOC dynamics, nitrogen and phosphorus cycling, respiration, enzyme activities and EMF sensitivity analyses. The implication would be to determine whether the increased EMF observed in Chapter 4 represents short-

term functional stimulation, true functional resilience, or an early warning of altered carbon and nutrient cycling (Allison and Martiny, 2008; Delgado-Baquerizo et al., 2016).

Finally, future research should place greater emphasis on comparability across microplastic ecotoxicology studies. Standardised reporting of particle characteristics, exposure units, weathering methods, biological endpoints and statistical outputs would improve the interpretation and integration of results across different experimental systems. In particular, studies should report polymer type, particle size distribution, morphology, weathering status, mass concentration, particle number concentration and exposure duration in a consistent manner. More harmonised experimental designs, including common reference materials, comparable exposure scenarios and transparent reporting of null findings, would help distinguish particle-specific effects from methodological variation. This would allow future research to move beyond isolated experimental observations toward more robust cross-study synthesis of how particle properties, exposure conditions and biological contexts shape microplastic responses.

5.9 Conclusion

This thesis investigated how PS micro- and nanoplastics influence freshwater organisms and sediment-associated functional processes, using a combination of zebrafish experiments, an investigation of weathering-induced alterations in microplastic properties, and a simplified multi-species freshwater-sediment microcosm. Together, these studies provide a cross-scale perspective on how PS particles may influence stress-related biological responses and sediment-associated ecosystem processes under controlled laboratory conditions.

One of the important findings is that UV aging and mechanical weathering modified PS particle properties and altered their biological effects. UV aging altered the surface chemistry and morphology of 500 nm PS nanoplastics and was associated with stronger oxidative, immune-related, gut microbiota and gut-liver metabolic responses in adult zebrafish than pristine PS nanoplastics. Mechanical weathering produced rougher and more irregular PS microplastics and was associated with stronger stress-related responses in *V. spiralis* and *L. stagnalis*, as well as changes in sediment properties, enzyme activities

and respiration. These findings support the importance of incorporating weathered or aged plastic particles into micro- and nanoplastic assessments.

Across organismal and ecosystem levels, exposure to PS particles was linked with changes in oxidative stress, metabolic and functional indicators. These responses may reflect biological adjustment under exposure pressure, including increased demand for stress defence, maintenance of physiological homeostasis and stimulation of sediment microbial processes. In fish, oxidative stress and metabolic responses were observed together with reproductive changes, suggesting possible trade-offs between stress response and reproductive investment. In the microcosm, increased sediment respiration and β-glucosidase activity suggested stimulation of carbon-related microbial processes.

The research also revealed that adult female zebrafish showed broader biological responses than males. These responses included oxidative stress, gut microbiota disturbance, liver or gut-liver metabolic alteration and reproduction-related changes. This suggests that sex is an important biological variable when assessing effects of micro- and nanoplastics on zebrafish. The results indicate that female zebrafish may experience stronger physiological trade-offs under the tested exposure conditions, potentially due to reproductive energy demand, endocrine regulation, hepatic metabolism or gut-liver interactions.

At the ecosystem scale, the microcosm experiment showed that ecosystem multifunctionality increased under PS microplastic exposure, despite stress-related responses in *V. spiralis* and *L. stagnalis*. This increase was mainly associated with elevated dissolved organic carbon, β-glucosidase activity and sediment respiration, suggesting enhanced microbial carbon processing. However, this may reflect short-term functional stimulation or compensation, rather than improved ecosystem health or long-term ecological resilience.

In summary, this thesis advances understanding of PS micro- and nanoplastics effects by integrating biological sex, particle transformation and sediment-associated ecosystem functioning. The findings highlight the need to consider transformed particles, gender-specific responses and cross-compartment interactions when assessing freshwater microplastic impacts.

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Appendix I. Supplementary materials for Chapter 2

Text S2.1. 16S rRNA sequencing

Extraction of genome DNA: The CTAB/SDS method was used to extract the total genome DNA in samples. DNA concentration and purity were monitored on 1% agarose gels. According to the concentration, DNA was diluted to 1ng/μL with sterile water.

Amplicon Generation: 16S rRNA genes in distinct regions (16S V3-V4) were amplified with specific primer and barcodes. Primer sequences are shown in Table S2.1 (Qian et al., 2021). All PCR mixtures contained 15 μL of Phusion® High-fidelity PCR Master Mix (New England Biolabs), 1 μM of each primer and 10ng target DNA. Cycling conditions consisted of a first denaturation step at 98°C for 1 min, followed by 30 cycles at 98°C (10s), 50°C(30s) and 72°C(30s) and a final 5 min extension at 72°C.

PCR Products quantification and qualification: An equal volume of 1X loading buffer (contained SYBR green) was mixed with PCR products and electrophoresis was performed on 2% agarose gel for DNA detection. The PCR products were mixed in equal proportions, and then Universal DNA PCR Purification Kit (TianGen, China, Catalog #: DP214) was used to purify the mixed PCR products.

Library preparation and sequencing: Following the manufacturer's recommendations, sequencing libraries were generated with NEB Next Ultra DNA Library Prep Kit for Illumina (NEB, USA, Catalog #: E7370L). The library quality was assessed on the Agilent 5400 system (Agilent, USA) and quantified by real-time PCR (1.5nM). Finally, the library was sequenced on an Illumina NovaSeq platform and 250 bp paired-end reads were generated.

Data analysis: The raw reads were merged using FLASH (V1.2.7) and then filtered to remove low-quality reads and chimera sequences using QIIME (v1.9.1) and UCHIME. The effective tags were then clustered by UPARSE to Operational Taxonomic Units (OTUs) according to 97% similarity. For each representative sequence, the Silva Database was used based on Mothur algorithm to annotate taxonomic information and count the community composition of classification level (e.g., phylum and genus). Alpha diversity and beta diversity were calculated with QIIME. Gut microbiota functions were predicted

using Tax4Fun, and pathway information were performed on Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.kegg.jp/).

Text S2.2. Liver metabolomics

Sample preparation

Sample was accurately weighed into a 2 mL centrifuge tube with 1000 μL tissue extract (75% 9:1 methanol: chloroform, 25% H2O) and 3 steel balls. Then the sample was ground at 50 Hz for 60 s in tissue grinder twice. After grinding, sample was sonicated for 30 min and cooled in an ice bath for 30 min. The sample was centrifuged at 12,000 rpm and 4 °C for 10 min. The supernatant was transferred to a new 2 mL centrifuge tube, vacuum condensed and dried for 9 hours (Eppendorf Concentrator plus). 200 μL 50% acetonitrile solution prepared with 2-Amino-3-(2-chloro- phenyl)-propionic acid (4 ppm) (stored at 4 ℃) was added to re dissolve the sample. The supernatant was filtered by 0.22 μm polytetrafluoroethylene (PTFE) membrane and transferred into the detection bottle for LC-MS detection.

Liquid chromatography conditions

The LC analysis was performed on a Vanquish UHPLC System (Thermo Fisher Scientific, USA). Chromatography was carried out with an ACQUITY UPLC ® HSS T3 (150×2.1 mm, 1.8 μm) (Waters, Milford, MA, USA). The column maintained at 40 ℃. The flow rate and injection volume were set at 0.25 mL/min and 2 μL, respectively. For LC-ESI (+)-MS analysis, the mobile phases consisted of (A) 0.1% formic acid in acetonitrile (v/v) and (B) 0.1% formic acid in water (v/v). Separation was conducted under the following gradient: 0~1 min, 2% A; 1~9 min, 2%~50% A; 9~12 min, 50%~98% A 12~13.5 min, 98% A; 13.5~14 min, 98%~2% A; 14~20 min, 2% A. For LC-ESI (-)-MS analysis, the analytes were carried out with (C) acetonitrile and (D) ammonium formate (5mM). Separation was conducted under the following gradient: 0~1 min, 2% C; 1~9 min, 2%~50% C; 9~12 min, 50%~98% C; 12~13.5 min, 98% C; 13.5~14 min, 98%~2% C; 14~17 min, 2% C.

Mass spectrum conditions

Mass spectrometric detection of metabolites was performed on Orbitrap Exploris 120

(Thermo Fisher Scientific, USA) with ESI ion source. Simultaneous MS1 and MS/MS (Full MS-ddMS2 mode, data-dependent MS/MS) acquisition was used. The parameters were as follows: sheath gas pressure, 30 arb; aux gas flow, 10 arb; spray voltage, 3.50 kV and -2.50 kV for ESI (+) and ESI (-), respectively; capillary temperature, 325 ℃; MS1 range, m/z 100-1000; MS1 resolving power, 60000 FWHM; number of data dependent scans per cycle, 4; MS/MS resolving power, 15000 FWHM; normalized collision energy, 30%; dynamic exclusion time, automatic.

Data processing and multivariate analysis

The raw data were firstly converted to mzXML format by MSConvert in the ProteoWizard software package (v3.0.8789) and processed using XCMS for feature detection, retention time correction and alignment. The metabolites were identified by accuracy mass (< 30 ppm) and MS/MS data which were matched with HMDB (http://www.hmdb.ca), massbank (http://www.massbank.jp/), LipidMaps (http://www.lipidmaps.org), mzcloud (https://www.mzcloud.org) and KEGG (http://www.genome.jp/kegg/). The robust LOESS signal correction (QC-RLSC) was applied for data normalization to correct for any systematic bias. After normalization, only ion peaks with relative standard deviations (RSDs) less than 30 % in QC were kept to ensure proper metabolite identification.

The Ropls software was used for all multivariate data analyses and modeling. Data were mean-centered using scaling. Models were built on principal component analysis (PCA), orthogonal partial least-square discriminant analysis (PLS-DA) and partial least-square discriminant analysis (OPLS-DA). The metabolic profiles could be visualized as a score plot, where each point represents a sample. The corresponding loading plot and S-plot were generated to provide information on the metabolites that influence clustering of the samples. All of the models evaluated were tested for over fitting with methods of permutation tests. The descriptive performance of the models was determined by R2X (cumulative) (perfect model: R2X (cum) = 1) and R2Y (cumulative) (perfect model: R2Y (cum) = 1) values while their prediction performance was measured by Q2 (cumulative) (perfect model: Q2 (cum) = 1) and a permutation test. The permuted model should not be able to predict classes: R2 and Q2 values at the Y-axis intercept must be lower than those

of Q2 and the R2 of the non-permuted model. OPLS-DA allowed the determination of discriminating metabolites using the variable importance on projection (VIP). The variable importance projection (VIP) produced by OPLS-DA fold change (FC), was applied to discover the contributable-variable for classification. Finally, P values < 0.05 and VIP values > 1 were considered to be statistically significant metabolites.

Pathway analysis

Differential metabolites were subjected to pathway analysis by MetaboAnalyst, which combines results from powerful pathway enrichment analysis with the pathway topology analysis. The identified metabolites in metabolomics were then mapped to the KEGG pathway for biological interpretation of higher-level systemic functions. The metabolites and corresponding pathways were visualized using KEGG Mapper tool.

Table S2.1. Primers of the selected genes in this study.

Primer pairs	Sequence (5'—3')
341F	CCTAYGGGRBGCASCAG
806R	GGACTACNNGGGTATCTAAT

Table S2.2. Statistical results and exact p-values for the biological endpoints measured in Chapter 2.

Figure	Index	Comparison	P value
2.2	SOD	F-1μm vs F-control	0.047
2.2	MDA	F-1μm vs F-control	0.007
2.10	$\delta^{13}C$	F-1μm vs F-control; M-1μm vs M-control	0.03; 0.048
2.10	$\delta^{15}N$	F-1μm vs F-control; M-1μm vs M-control	0.002; 0.028
2.10	E2	F-1μm vs F-control	0.000028
2.10	T	F-1μm vs F-control	0.003
2.10	E2/T	F-1μm vs F-control; M-1μm vs M-control	0.000478; 0.016
2.10	Eggs	♀1μm×♂ck vs ♀ck×♂ck; ♀1μm×♂1μm vs ♀ck×♂ck	0.022; 0.049

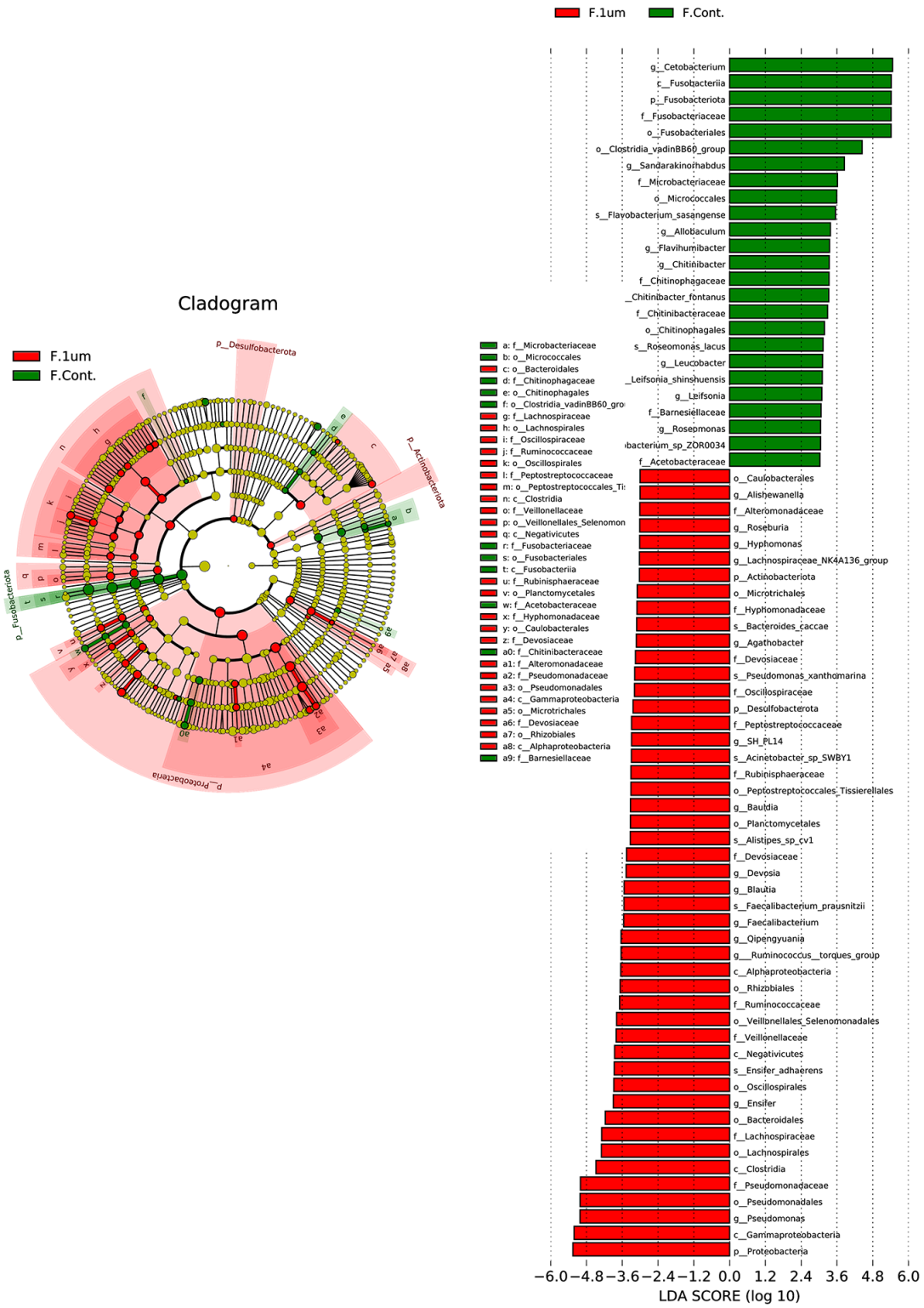


Figure S2.1. Linear discriminant analysis Effect Size (LEfSe) analysis of gut bacteria of female zebrafish affected by polystyrene (PS) microplastics. The circles in the cladogram represent taxa from kingdom to genus (from inside to outside).

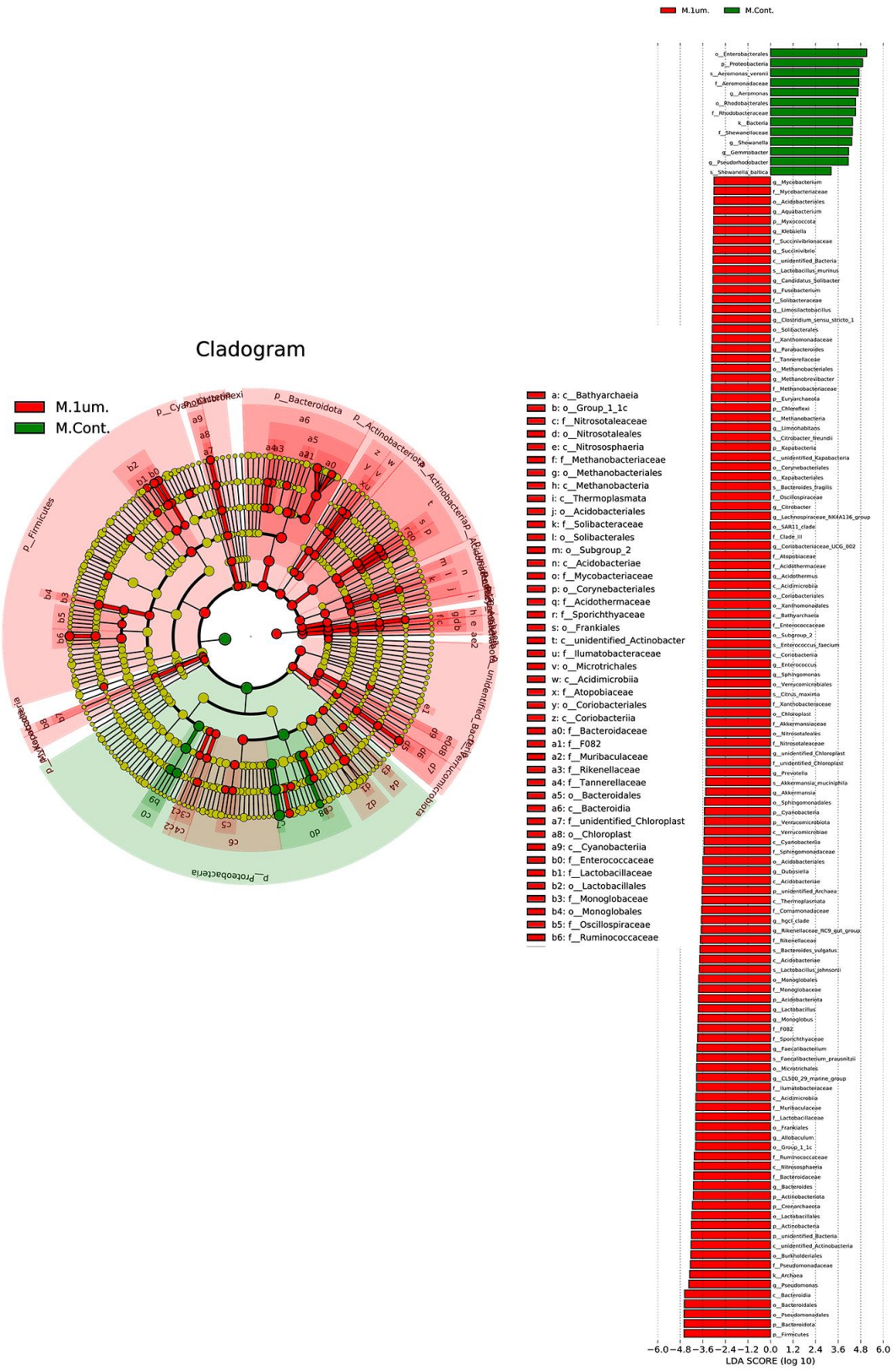


Figure S2.2. Linear discriminant analysis Effect Size (LEfSe) analysis of gut bacteria of male zebrafish affected by polystyrene (PS) microplastics. The circles in the cladogram represent taxa from kingdom to genus (from inside to outside).

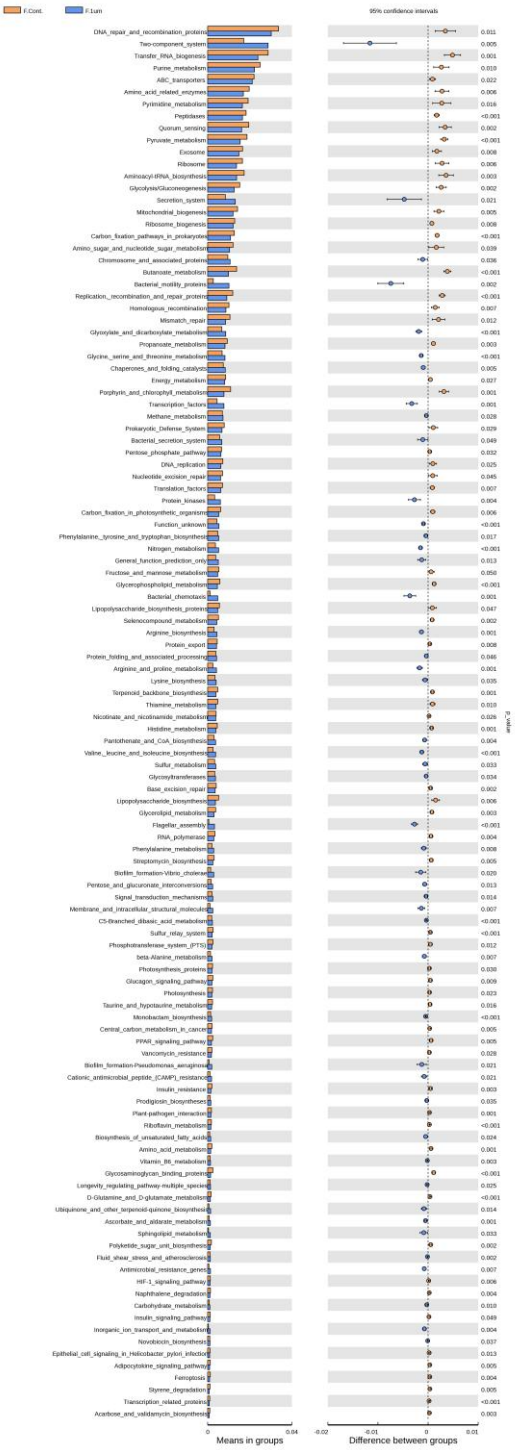


Figure S2.3. Welch's t-test analysis of predicted function (level 3) of zebrafish gut microbiota in female groups by Tax4Fun analysis.

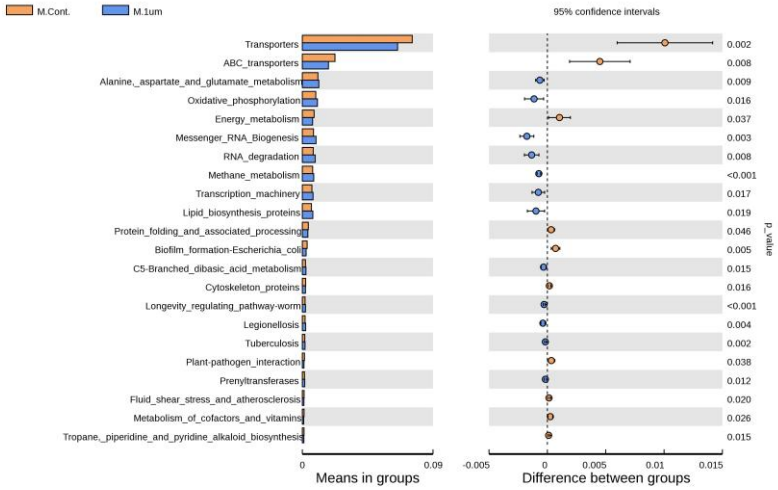


Figure S2.4. Welch's t-test analysis of predicted function (level 3) of zebrafish gut microbiota in male groups by Tax4Fun analysis.

References

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Appendix II. Supplementary materials for Chapter 3

Text S3.1. Skin mucus sample collection

Twenty zebrafish per group were transferred to a sterile polyethylene bag containing 5 mL NaCl (100 mM) after being anaesthetized and washed. Then, fish were gently rubbed back and forth on the bag for 2-3 min to collect the mucus. The collected samples were centrifuged (12, 000 rpm, 30 min, 4 ℃) and the supernatant was filtered using a 0.22 μm filter for further analysis.

Text S3.2. IBR analysis

For the IBRv2 calculation, individual biomarker data (X_i) are compared to a mean reference value (X_0) and a log transformation is applied to reduce variance.

$$Y_i = \log\left(\frac{X_i}{X_0}\right) \tag{3.1}$$

In a next step, the general mean (μ) and standard deviation (s) of Y_i were computed as previously described by Beliaeff and Burgeot (2002), and Y_i is standardized.

$$Z^i = \frac{Y_i - \mu}{\sigma} \tag{3.2}$$

To create a basal line centred on 0 and to represent biomarker variation according to this basal line, the mean of standardized biomarker response (Z_i) and mean of reference biomarker data (Z_0) are used to define a biomarker deviation index (A).

$$A = Z_i - Z_0 \tag{3.3}$$

To obtain an integrated multi-biomarker response named IBRv2, the absolute value of A parameters calculated for each biomarker in each investigated site are summed.

$$IBRv2 = \sum |A| \tag{3.4}$$

Text S3.3. 16S rRNA sequencing

Extraction of genome DNA - The CTAB/SDS method was used to extract the total genome DNA in samples. DNA concentration and purity were monitored on 1% agarose gels. DNA concentration was then diluted to 1ng/μL with sterile water.

Amplicon generation - 16S rRNA genes in distinct regions (16S V3-V4) were

amplified with specific primer and barcodes. All PCR mixtures contained 15 μL of Phusion® High-fidelity PCR Master Mix (New England Biolabs), 1 μM of each primer and 10ng target DNA. Cycling conditions consisted of a first denaturation step at 98°C for 1 min, followed by 30 cycles at 98°C (10s), 50°C(30s) and 72°C(30s) and a final 5 min extension at 72°C.

PCR product quantification and qualification - An equal volume of 1X loading buffer (contained SYBR green) was mixed with PCR products and electrophoresis was performed on 2% agarose gel for DNA detection. The PCR products were mixed in equal proportions, and then the Universal DNA PCR Purification Kit (TianGen, China, Catalog #: DP214) was used to purify the mixed PCR products.

Library preparation and sequencing: following the manufacturer's recommendations, sequencing libraries were generated with NEB Next Ultra DNA Library Prep Kit for Illumina (NEB, USA, Catalog #: E7370L). The library quality was assessed on the Agilent 5400 system (Agilent, USA) and quantified by real-time PCR (1.5nM). Finally, the library was sequenced on an Illumina NovaSeq platform and 250 bp paired-end reads were generated.

Data analysis - The raw reads were merged using FLASH (V1.2.7) and then filtered to remove low-quality reads and chimera sequences using QIIME (v1.9.1) and UCHIME. The effective tags were then clustered by UPARSE to Operational Taxonomic Units (OTUs) according to 97% similarity. For each representative sequence, the Silva Database was used based on Mothur algorithm to annotate taxonomic information and count the community composition by classification level (e.g., phylum and genus). Alpha diversity and beta diversity were calculated with QIIME. Gut microbiota functions were predicted using PICRUSt2, and pathway information was obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.kegg.jp/). The functional abundance between the two sets of samples was further compared using Metastats differential analysis based on the findings of the PICRUSt2 functional classification.

The sequencing data were deposited in the NCBI database (BioProject accession: PRJNA1265363).

Text S3.4. Gut and liver metabolomics

Sample preparation and extraction - The tissues (100mg) were quickly frozen in liquid nitrogen and ground into fine powder with a pestle and mortar. The tissue samples with 100 μL of ice-cold H_2O and five ceramic beads were homogenized using a homogenizer for 60s. 400 μL methanol/acetonitrile (1:1, v/v) were added to the homogenized solution for metabolite extraction. The solutions were sonicated for 30 min at 4°C. After centrifugation (20 min, 12,000 rpm, 4°C), the supernatant was transferred to clean plastic microtubes. The samples were freeze-dried and stored at -20°C until analysis. For UHPLC–ESI–MS/MS analysis, the samples were dissolved in 200 μL of 30% ACN (vol/vol) and transferred to insert-equipped vials.

UPLC conditions - The sample extracts were analysed using an UPLC – Orbitrap - MS system (UPLC, Vanquish; MS, HFX). The analytical conditions were as follows, UPLC: column, Waters HSS T3 （100*2.1 mm, 1.8μm; column temperature, 40 ℃; flow rate, 0.3 mL/min; injection volume, 2 μL; solvent system, water (0.1% Acetic acid) (A): acetonitrile (0.1% Acetic acid) (B); gradient program, 0 min, 0% B; 1min, 0% B; 9 min, 95% B; 13 min, 95% B; 13.1 min, 0% B; 17 min, 0%B.

LC-MS/MS analysis - HRMS data were recorded on a Q Exactive HFX Hybrid Quadrupole Orbitrap mass spectrometer equipped with a heated ESI source (Thermo Fisher Scientific) utilizing the full-ms-ddMS2 MS acquisition methods. The ESI source parameters were set as follows: spray voltage, -2.8 kV/3.0 kV; sheath gas pressure, 40 arb; aux gas pressure, 10 arb; sweep gas pressure, 0 arb; capillary temperature, 320℃; and aux gas heater temperature, 350℃.

Data analysis and interpretation -The raw MS data were acquired on the Q-Exactive using Xcalibur 4.1 (Thermo Scientific), and processed using Progenesis QI (Waters Corporation， Milford, USA). Quantified data were output into Excel format. Data were analysed by R package, where it was subjected to multivariate data analysis, including principal component analysis (PCA) and orthogonal partial least-squares discriminant analysis (OPLS-DA). Seven-fold cross-validation and response permutation testing were used to evaluate the robustness of the model. The variable importance in the projection (VIP) value of each variable in the OPLS-DA model was calculated to indicate its

contribution to the classification. Metabolites with a VIP value >1 were further included in a Student's t-test at univariate level to measure the significance of each metabolite: p values < 0.05 were considered as statistically significant.

Bioinformatics analysis - (1) PCA was performed by statistics function *ropls* within R (www.r-project.org). The data were unit variance scaled before unsupervised PCA. (2) Hierarchical Cluster Analysis (HCA) results for samples and metabolites were presented as heatmaps with dendrograms, while Pearson correlation coefficients (PCC) between samples were calculated by the corfunction in R and presented only as heatmaps. Both HCA and PCC were carried out using the R package pHeatmap. (3) Differential metabolites were selected. Significantly regulated metabolites between groups were determined by VIP > 1 and FC (fold change) > 1.5 or FC (fold change) < 0.67. VIP values were extracted from the OPLS-DA results, which also contain score plots and permutation plots, generated using R package *ropls*. The data were log transformed (log_2) and mean centred before OPLS-DA. In order to avoid overfitting, a permutation test (200 permutations) was performed. (4) KEGG annotation and enrichment analysis was undertaken. Identified metabolites were annotated using the KEGG Compound database (http://www.kegg.jp/kegg/compound/), and annotated metabolites were then mapped to the KEGG Pathway database (http://www.kegg.jp/kegg/pathway.html) .

Table S3.1. Statistical results and exact p-values for the biological endpoints measured in Chapter 3.

Figure	Index	Comparison	P value
3.3	IgM	FP vs F-control; FA vs F-control	0.00026; 0.000008
3.3	LPS	FA vs F-control; MA vs M-control	0.019; 0.004
3.3	TG	FA vs F-control; MA vs M-control	0.009; 0.037
3.3	ROS	FA vs F-control	0.042
3.3	SOD	FA vs F-control; MA vs M-control	0.028; 0.005
3.3	MDA	MA vs M-control	0.004

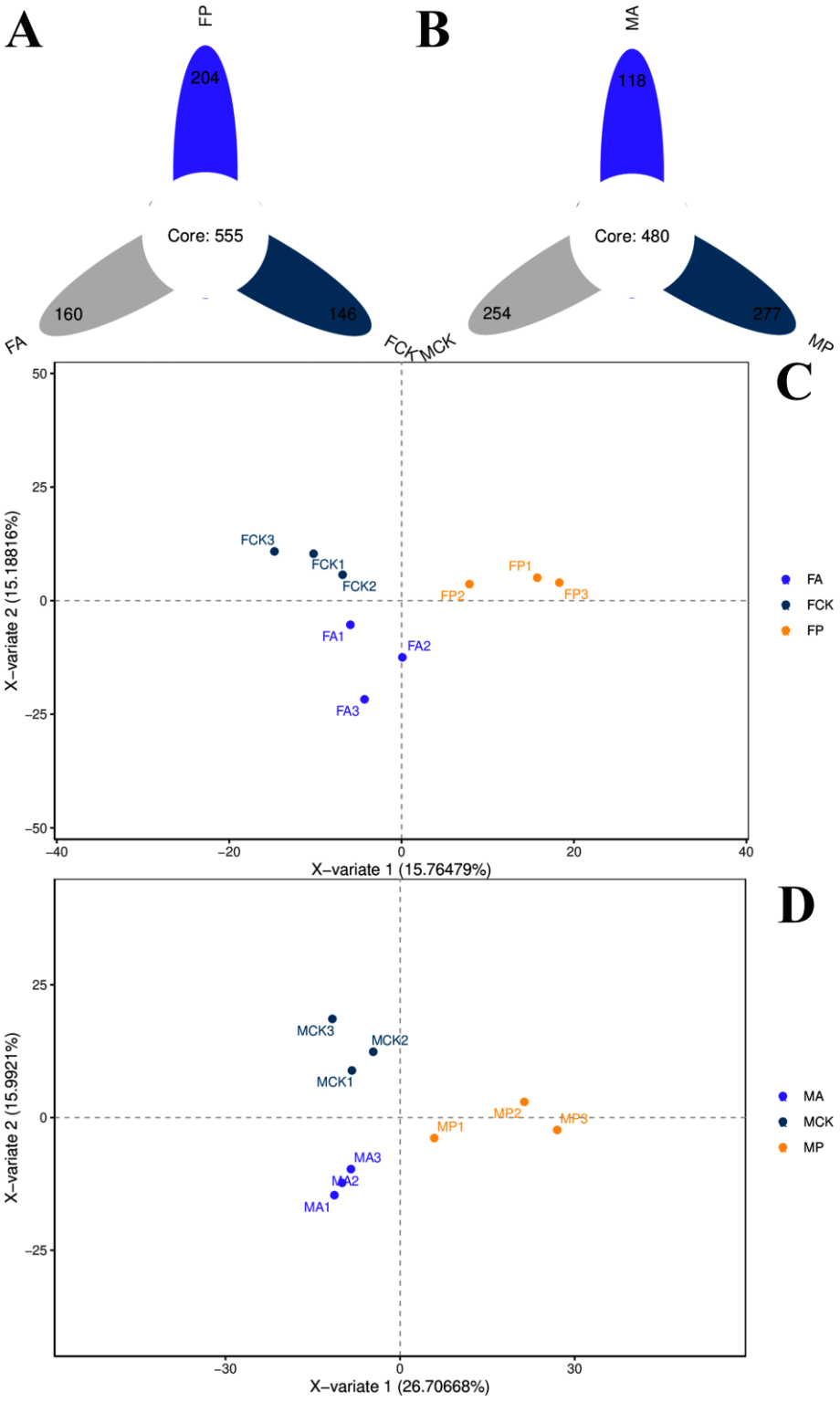


Figure S3.1. Venn diagram of bacterial OTUs unique within the gut microbiota of zebrafish after exposure to pristine and UV-aged polystyrene (PS) nanoplastics for 21 days. A, female groups; B, male groups. Partial least squares discrimination analysis (PLS-DA) of the gut microbiota beta-diversity of the control and PS nanoplastic treated groups, C, female groups; D, male groups.

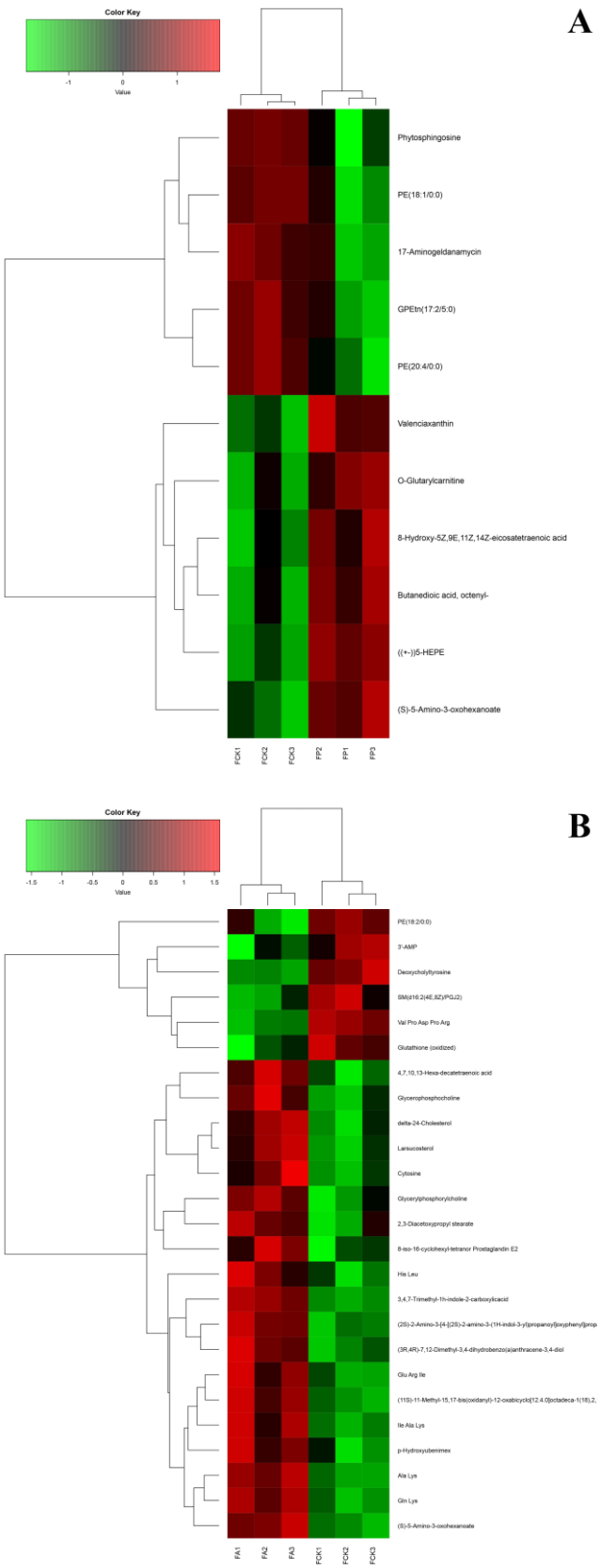


Figure S3.2. Heatmap of the differential metabolites in female zebrafish gut after pristine and UV-aged polystyrene (PS) nanoplastics exposure for 21 days, A, F-control group and FP group; B, F-control group and FA group.

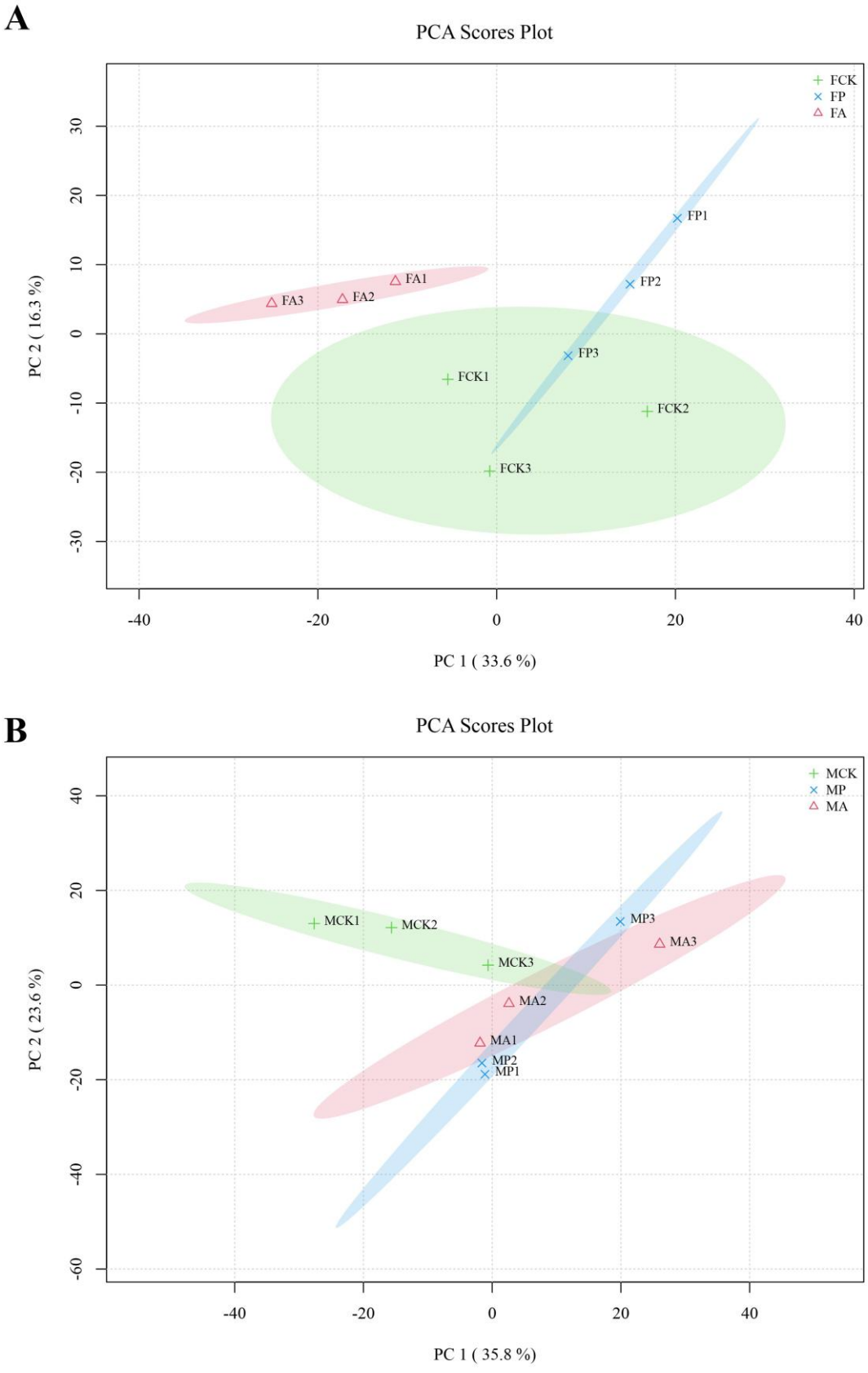


Figure S3.3. Principal components analysis (PCA) of the liver metabolites of control, pristine and UV-aged polystyrene (PS) nanoplastics groups, A, female groups; B, male groups.

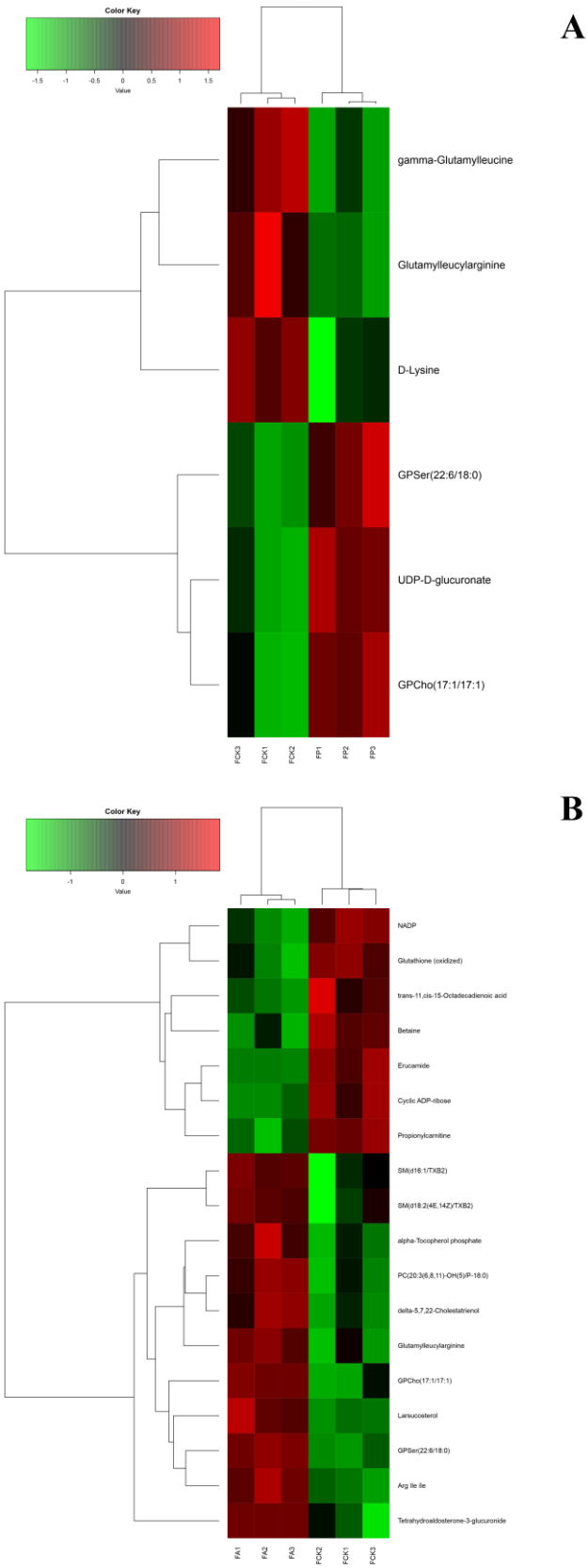


Figure S3.4. Heatmap of the differential metabolites in liver after pristine and UV-aged polystyrene (PS) nanoplastic exposure for 21 days, A, F-control group and FP group; B, F-control group and FA group;

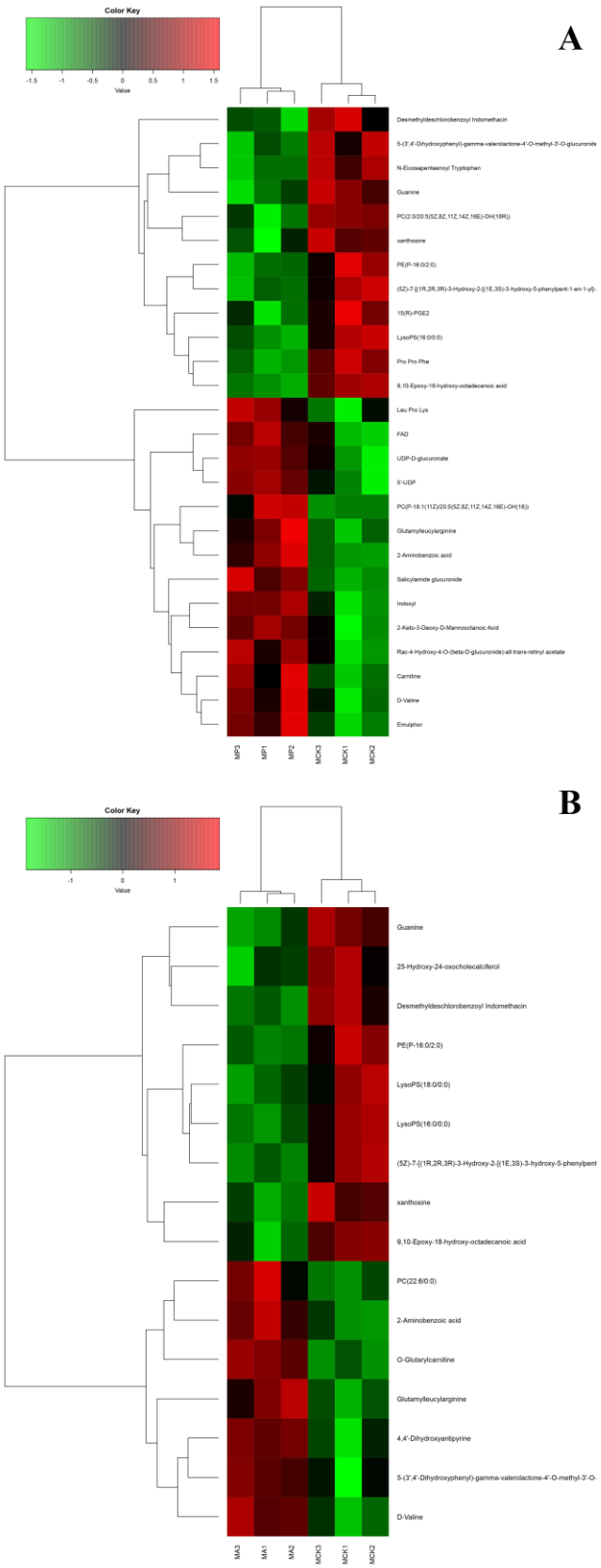


Figure S3.5. Heatmap of the differential metabolites in liver after pristine and UV-aged polystyrene (PS) nanoplastics exposure for 21 days, A, M-control group and MP group; B, M-control group and MA group.

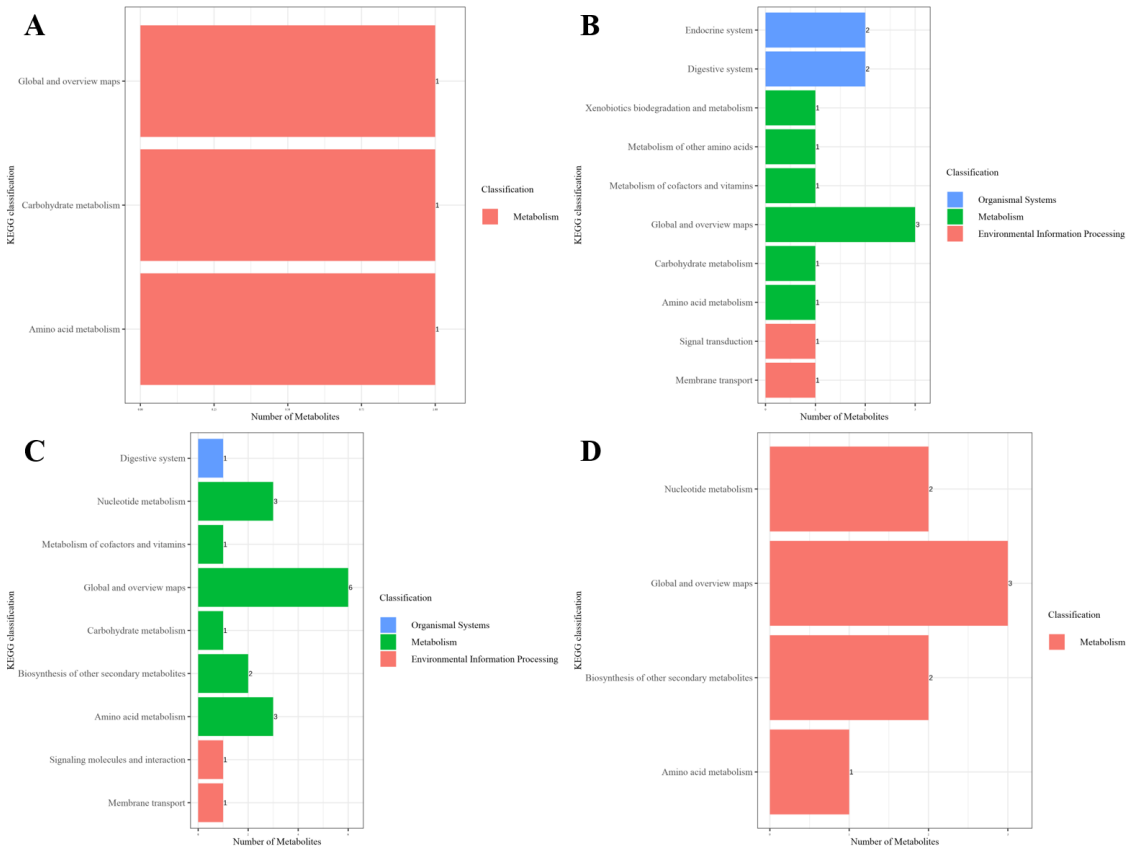


Figure S3.6. KEGG pathway annotation in zebrafish liver after pristine and UV-aged polystyrene (PS) nanoplastic exposure, A, FP group; B, FA group; C, MP group; D, MA group.

References

Beliaeff, B., & Burgeot, T. (2002). Integrated biomarker response: A useful tool for ecological risk assessment. *Environ Toxicol Chem, 21*(6), 1316-1322.

Appendix III. Supplementary materials for Chapter 4

Text S4.1 IBR analysis

Individual biomarker data (X_i) are compared to a mean reference value (X_0) and a log transformation is applied to reduce variance.

$$Y_i = \log\left(\frac{X_i}{X_0}\right) \tag{4.1}$$

In a next step, the general mean (μ) and standard deviation (s) of Y_i were computed as previously described by Beliaeff and Burgeot (Beliaeff & Burgeot, 2002), and Y_i is standardized:

$$Z^i = \frac{Y_i - \mu}{\sigma} \tag{4.2}$$

To create a basal line centred on 0 and to represent biomarker variation according to this basal line, the mean of standardized biomarker response (Z_i) and mean of reference biomarker data (Z_0) are used to define a biomarker deviation index (A):

$$A = Z_i - Z_0 \tag{4.3}$$

To obtain an integrated multi-biomarker response named IBRv2, the absolute value of A parameters calculated for each biomarker in each investigated site are summed:

$$IBRv2 = \sum |A| \tag{4.4}$$

Table S4.1. Statistical results and exact p-values for the biological endpoints measured in Chapter 4.

Figure	Index	Comparison	P value
4.2	TC	P-MPs vs Control; W-MPs vs Control	0.005; 0.008
4.2	TOC	P-MPs vs Control; W-MPs vs Control	0.001;0.003
4.3	pH	P-MPs vs Control; W-MPs vs Control	0.029; 0.001
4.3	TC	P-MPs vs Control; W-MPs vs Control	0.002;0.015
4.3	DOC	P-MPs vs Control; W-MPs vs Control	0.048;0.023
4.3	Urease	W-MPs vs Control	0.005
4.3	BG	P-MPs vs Control; W-MPs vs Control	0.000209; 0.022
4.3	Respiration rate	P-MPs vs Control; W-MPs vs Control	0.005; 0.026
4.4	Chl-a	W-MPs vs Control	0.009
4.4	carotenoids	P-MPs vs Control; W-MPs vs Control	0.023; 0.015
4.7	SOD	W-MPs vs Control	0.019
4.7	MDA	W-MPs vs Control	0.043
4.8	Soluble sugar	W-MPs vs Control	0.008
4.8	Amino acids	W-MPs vs Control	0.023
4.8	Starch	W-MPs vs Control	0.038
4.9	SOD	W-MPs vs Control	0.033
4.10	$Na^{+}K^{+}$ATPase	W-MPs vs Control	0.002
4.10	$Ca^{2+}Mg^{2+}$ATPase	W-MPs vs Control	0.006
4.10	Caspase-3	W-MPs vs Control	0.023
4.11	EMF	P-MPs vs Control; W-MPs vs Control	0.00037; 0.009

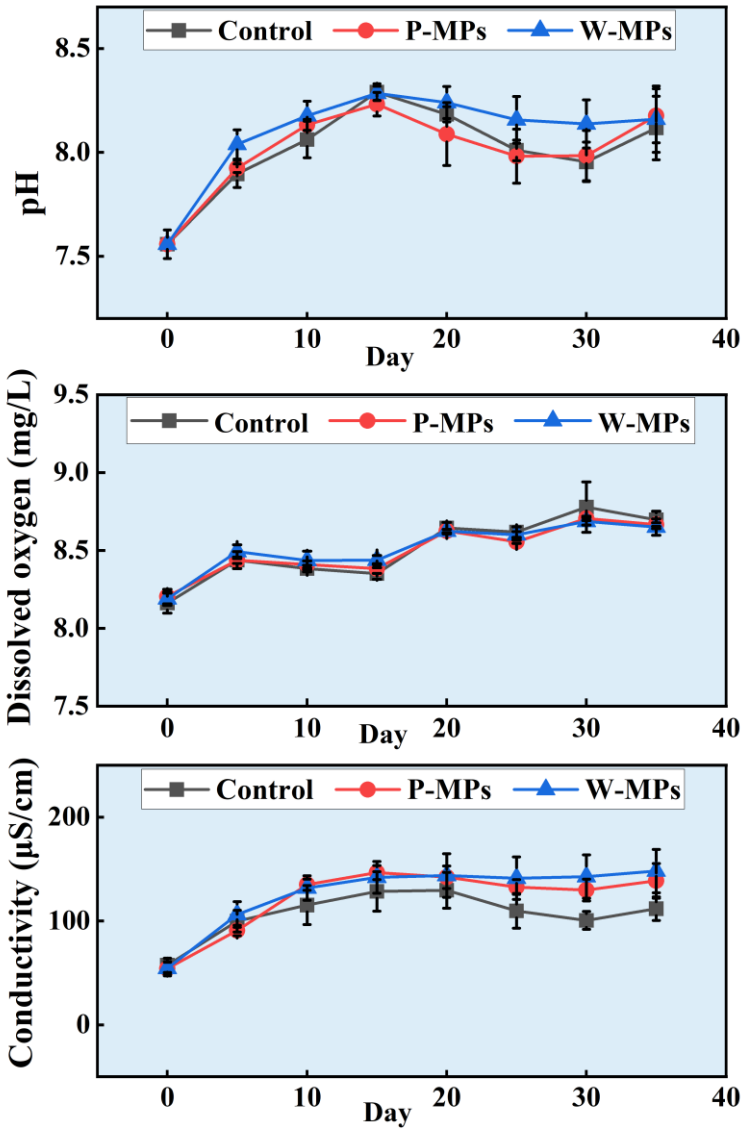


Figure S4.1. Alteration of pH (A), dissolved oxygen (B) and conductivity (C) of overlying water during 5-week exposure.

References

Beliaeff, B., & Burgeot, T. (2002). Integrated biomarker response: A useful tool for ecological risk assessment. *Environmental Toxicology and Chemistry, 21*(6), 1316-1322.