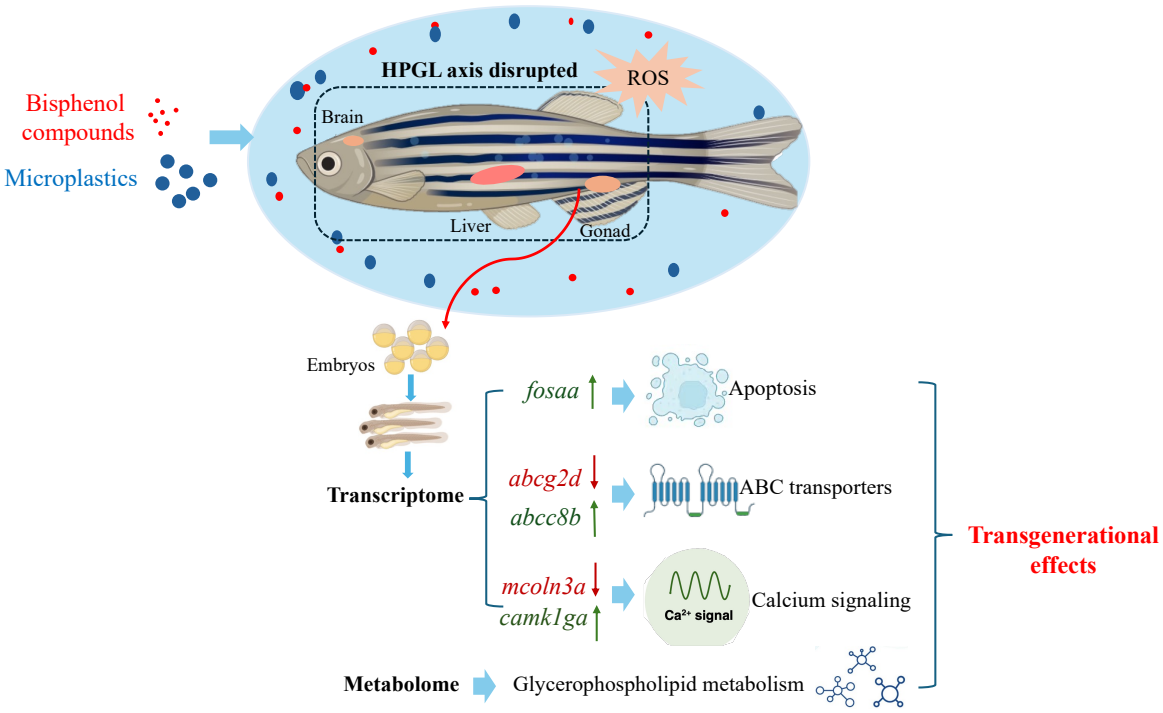
The toxicity and mechanism of combined exposure to microplastics and bisphenol compounds on zebrafish



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Abstract

This study systematically investigates the combined effects of bisphenol compounds (BPA and BPS) and microplastics (MP) on zebrafish, focusing on bioaccumulation, reproductive toxicity and transgenerational impacts. Through a multi-disciplinary approach incorporating analytical chemistry, molecular biology and toxicology, the thesis explores how the co-exposure of these pollutants leads to enhanced bioavailability, hormonal disruption, tissue damage and long-term ecological risks. A sensitive UPLC-MS/MS method was developed for quantifying BPA and BPS in zebrafish tissues, focusing on their bioaccumulation in the presence of MP. Microplastics significantly enhance the bioavailability of bisphenols, leading to higher concentrations in key organs such as liver and intestines. This highlights the role of MP as vectors for hydrophobic pollutants, exacerbating their toxic effects and providing the basis for understanding the heightened risks posed by pollutant mixtures in aquatic environments. Also, the oxidative stress and cellular damages caused by combined exposure to bisphenols and MP were examined in adult zebrafish. Significant downregulation of key antioxidant enzymes and increased apoptosis in liver and intestinal tissues were found, indicating severe oxidative stress. This cellular damage contributed to reproductive dysfunction, providing mechanistic insight into how combined pollutants affect aquatic species at the molecular and cellular levels. Moreover, extended analysis were performed to explore the transgenerational effects of bisphenol and MP co-exposure. The reproductive toxicity was observed in adult zebrafish, including gonadal damages and hormone imbalances to be transmitted to the F1 generation. Transcriptomic and metabolomic analyses of the offspring revealed significant disruptions in apoptosis, energy metabolism and developmental pathways. These findings highlight the long-term risks of combined pollutant exposure, not only to individual organisms but also to population dynamics across generations. This research underscores the compounded hazards posed by the combined exposure to bisphenols and microplastics, as these pollutants together result in more severe and widespread ecological damage than when studied individually. The findings highlight the amplified toxic effects, particularly reproductive dysfunction and oxidative stress, which not only affect the exposed organisms but also have significant transgenerational impacts. The research provides new insights into the long-term risks of environmental pollutant mixtures, demonstrating the need for future studies to consider the cumulative effects of multiple contaminants. These results offer a critical foundation for advancing toxicology research and contribute to shaping more comprehensive regulatory frameworks that address the persistent and heritable dangers of such pollutants in aquatic ecosystems.

Keywords: Bisphenol compounds, microplastics, toxicity, transgenerational effects

Résumé

Cette étude examine systématiquement les effets combinés des composés bisphénols (BPA et BPS) et des microplastiques (MP) sur le poisson-zèbre, en se concentrant sur la bioaccumulation, la toxicité reproductive et les impacts transgénérationnels. Grâce à une approche multidisciplinaire intégrant la chimie analytique, la biologie moléculaire et la toxicologie, cette thèse explore comment la co-exposition à ces polluants entraîne une biodisponibilité accrue, des perturbations hormonales, des lésions tissulaires et des risques écologiques à long terme. Une méthode sensible a été développée par UPLC-MS/MS pour quantifier le BPA et le BPS dans les tissus de poisson-zèbre, en mettant l'accent sur leur bioaccumulation en présence de MP. Les MP augmentent considérablement la biodisponibilité des bisphénols, entraînant des concentrations plus élevées dans des organes clés comme le foie et les intestins. Cela met en évidence le rôle des MP en tant que vecteurs de polluants hydrophobes, exacerbant leurs effets toxiques et fournissant une base pour comprendre les risques accrus posés par les mélanges de polluants dans les environnements aquatiques. Aussi, le stress oxydatif et les dommages cellulaires causés par l'exposition combinée aux bisphénols et aux MP ont été examinés chez les poissons-zèbres adultes. Une réduction significative des enzymes antioxydantes clés et une augmentation de l'apoptose dans les tissus du foie et des intestins ont été observées, indiquant un stress oxydatif sévère. Ces dommages cellulaires ont contribué à la dysfonction reproductive, offrant une compréhension mécanistique de la manière dont les polluants combinés affectent les espèces aquatiques au niveau moléculaire et cellulaire. De plus, l'analyse pour explorer les effets transgénérationnels de la co-exposition aux bisphénols et aux MP a été réalisée. La toxicité reproductive observée chez les poissons-zèbres adultes, y compris les lésions gonadiques et les déséquilibres hormonaux, a été transmise à la génération F1. Les analyses transcriptomiques et métabolomiques des descendants ont révélé des perturbations significatives de l'apoptose, du métabolisme énergétique et des voies de développement. Ces résultats soulignent les risques à long terme de l'exposition combinée aux polluants, non seulement pour les organismes individuels mais aussi pour les dynamiques des populations à travers les générations. Cette recherche a permis de mettre en évidence les dangers aggravés de la co-exposition aux bisphénols et aux microplastiques, car ces polluants, ensemble, entraînent des dommages écologiques plus graves et plus répandus que lorsqu'ils sont étudiés séparément. Les résultats mettent en lumière les effets toxiques amplifiés, en particulier la dysfonction reproductive et le stress oxydatif, qui affectent non seulement les organismes exposés, mais aussi les générations futures de manière significative. Cette recherche fournit de nouvelles perspectives sur les risques à long terme des mélanges de polluants environnementaux, démontrant la nécessité de futures études prenant en compte les effets cumulatifs de multiples contaminants. Ces résultats offrent une base critique pour l'avancement des recherches en toxicologie et contribuent à l'élaboration de cadres

réglementaires plus complets pour aborder les dangers persistants et héréditaires de ces polluants dans les écosystèmes aquatiques.

Mots-clés: Composés bisphénols, microplastiques, toxicité, effets transgénérationnels

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List of acronyms

11-KT: 11-keto testosterone

ABC: ATP binding cassette

AchE: acetylcholinesterase

ARA: arachidonic acid

Ars: androgen receptors

BPA: bisphenol A

BPB: bisphenol B

BPF: bisphenol F

BPs: bisphenol compounds

BPS: bisphenol S

CAT: catalase

CNS: central nervous system

DEGs: differentially expressed genes

DNA: deoxyribonucleic acid

E2: estradiol

EDCs: endocrine-disrupting chemicals

ELISA: enzyme-linked immunosorbent assay

ERS: endoplasmic reticulum stress

ERs: estrogen receptors

ESI: electrospray ionization

FSH: follicle-stimulating hormone

GnRH: Gonadotropin-Releasing Hormone

GnRHRs: Gonadotropin-Releasing Hormone Receptors

GO: gene ontology

GPx: glutathione peroxidase

GRK: G protein-coupled receptor kinase

GSH: glutathione

HE: hematoxylin-eosin

HPG: hypothalamic-pituitary-gonadal

HPGL: hypothalamic-pituitary-gonadal-liver

HPT: hypothalamic-pituitary-thyroid

KEGG: Kyoto Encyclopedia of Genes and Genomes

LH: luteinizing hormone

LOD: limit of detection

LOQ: limit of quantification

MAPK: mitogen-activated protein kinase

MDA: malondialdehyde

MP: Microplastics

OPLS-DA: Orthogonal Partial Least Squares-Discriminant Analysis

PCA: Principal Component Analysis

PE-MP: Polyethylene microplastics

PP: Polypropylene

PS: Polystyrene

PS-NP: Polystyrene nanoplastics

PUFA: polyunsaturated fatty acid

RNA: Ribonucleic acid

ROS: reactive oxygen species

RSDs: relative standard deviations

SEM: standard error of the mean

SOD: superoxide dismutase

SPE: solid phase extraction

T: testosterone

UPLC: ultrahigh performance liquid chromatography system

UPLC-MS/MS: ultraperformance liquid chromatography-triple quadrupole mass spectrometer

VDCCs: Voltage-dependent calcium channels

VIP: Variable important in projection

VTG: vitellogenin

Chapter 1

Problem, research aim, thesis outline and experimental design

1. Problem

Microplastics represent a diverse class of pollutants that are pervasive in aquatic environments. A growing body of evidence indicates the widespread presence of microplastics in various aquatic systems, including rivers, lakes and estuaries. Similarly, bisphenol compounds, commonly used as key raw materials in epoxy resin production, are frequently detected in surface waters and have become significant environmental contaminants. Water ecosystems serve as crucial vectors for the transportation of bisphenol analogues, contributing to their prevalence. Given the ubiquitous distribution of both microplastics and bisphenol compounds in aquatic environment, their co-occurrence is highly probable. While the individual harmful effects of these pollutants on aquatic organisms have been extensively studied, there is a significant knowledge gap regarding their combined effects. Therefore, it is imperative to investigate the potential synergistic or additive impacts of microplastics and bisphenol compounds on aquatic fauna to better understand their composite ecological risks.

2. Research aim

This project aims to employ transcriptomic and metabolomic approaches to investigate the combined effects of bisphenol compounds and microplastics exposure on zebrafish, especially focusing on the transgenerational impacts on offspring and the mechanisms underlying cross-generational toxicity. The objectives include the following aspects:

(1) To build a determination method of bisphenol compounds in different zebrafish tissues, laying a solid foundation for further toxic analysis;
(2) To explore whether combined exposure to microplastics and bisphenol compounds produce different toxic effects compared to single exposure;
(3) To examine the mechanism of the toxic effects on zebrafish and the transgenerational effects after combined exposure of bisphenol compounds and microplastics.

3. Thesis outline

In Chapter 2, we provide a comprehensive overview covering the sources, distribution, and toxic effects on aquatic organisms of microplastics and bisphenol compounds. Additionally, it highlighted the combination of these two pollutants changes and even exacerbated their toxicological effects.

In Chapter 3, we successfully developed a sensitivity and accuracy ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method for the simultaneously determination two kinds of bisphenol compounds, bisphenol A (BPA) and bisphenol S (BPS). At the same time, the tissue distribution and accumulation of BPA and BPS alone or in combination with microplastics in zebrafish were investigated.

In Chapter 4, we investigated the effects of BPA and BPS exposure on oxidative damage in adult zebrafish, both individually and in combination with microplastics.

Furthermore, we explored potential mitigation strategies, such as melatonin treatment, to alleviate any observed adverse effects.

In Chapter 5, we examined the combined toxic effects of BPA and BPS, with or without the co-presence of MP, and integrated transcriptomics and metabolomics techniques, to clarify the underlying mechanisms of the effects on the offspring (F1 generation) after parental exposure on adult zebrafish.

4. Research roadmap

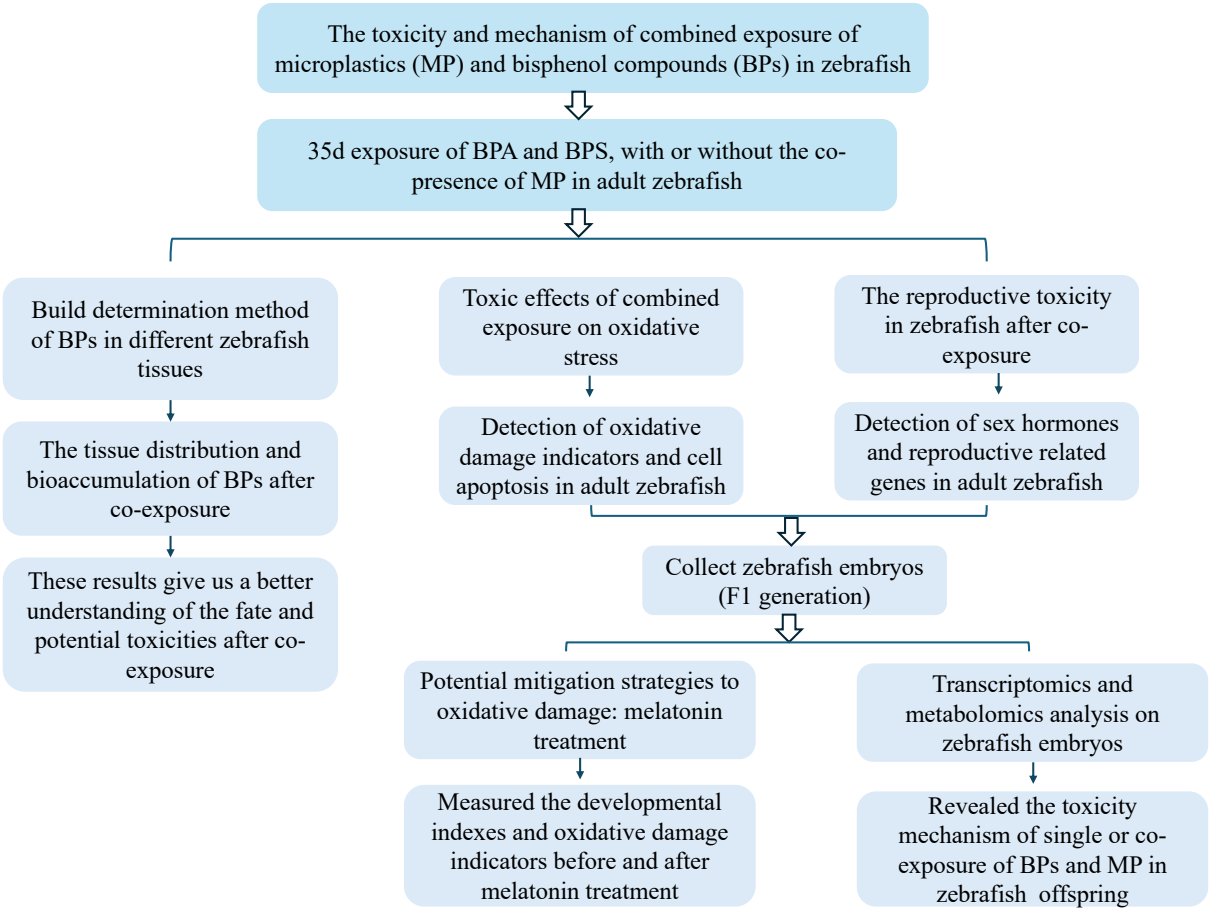

Figure 1-1. The research roadmap of this thesis.

Chapter 2

Microplastics and bisphenol compounds in aquatic ecosystem and their combined impact on fish health: a review

Abstract

Microplastics (MP) and bisphenol compounds (BPs) have emerged as pervasive environmental pollutants in aquatic ecosystems, raising global concern due to their potential to interact with biological systems and exacerbate toxic effects. This review explores the sources, distribution, and mechanisms through which these pollutants affect aquatic organisms, with a particular focus on fish. MP, classified as primary or secondary, pose physical and biological hazards to fish, including oxidative stress, immune disruption, reproductive failure, and neurotoxicity. BPs, commonly used in the production of plastics, are recognized as endocrine disruptors, impairing hormonal balance and reproductive functions in aquatic life. The co-occurrence of MP and BPs in aquatic environments amplifies their ecological risks, as MP can act as carriers for BPs, enhancing their bioavailability and toxicity. This synergistic toxicity is demonstrated in various fish species, leading to increased oxidative stress, endocrine disruption, and developmental abnormalities. A deeper understanding of the combined impacts of MP and BPs is critical for assessing their long-term effects on aquatic ecosystems and human health.

Keywords: Microplastics, bisphenol compounds, co-exposure, aquatic ecosystems, fish toxicity

1. Introduction

Microplastics (MP), detected in diverse ecosystems including marine environments, freshwater bodies, soil, atmosphere and even groundwater[1–4], as well as in drinking water and food, potentially posing a direct hazard to human health [5,6]. MP have become a matter of global significance due to their pervasive presence and the ability to interact with biological systems. However, there is still limited understanding of their long-term effects on both living organisms and non-living components[7–9]. Current data indicates that ecosystems contaminated by MP show reduced floral and faunal biomass, diminished productivity, impaired nitrogen cycling, decreased oxygen production, and lower carbon sequestration[10]. Also, the extensive dispersion of MP presents significant risks to the biodiversity of both aquatic and terrestrial organisms, pose a significant threat to essential species [11]. According to the literature, over 8 million tons of MP waste were discharged into ocean and freshwater environment from terrestrial sources[12]. These observations suggest that the impact of MP on ecological biomes is already evident. In soil, MP could alter the composition and metabolic processes of soil microbiota, which could, in turn, have significant repercussions on plant production[13]. The consumption of MP by aquatic species, including fish, turtles and seabirds, is extensively documented in scientific literatures[14,15] and these ingestions have detrimental effects on these organisms, such as disruptions in hormonal balance, lipid metabolism disorders, oxidative stress and neurotoxicity[16–18]. It should be noted that MP may be transmitted to humans through the food chain, as the consumption of contaminated seafood and agricultural products[9,19]. Once transmitted through the food chain[20–22], MP could accumulate in human bodies who are at the top of the food chain.

MP could accumulate and magnify as they transfer through trophic levels from herbivores to carnivores, eventually entering the human body[11,23,24]. Humans are exposed to MP through ingestion, inhalation, and dermal contact, with these particles having been detected in various bodily fluids, including urine, feces, blood, thrombi, and even placental tissues[25]. This alarming potential for MP to exacerbate pollution has led to growing concerns[26], therefore, gaining a more comprehensive and broader understanding of microplastics is essential[27,28] for addressing these challenges and developing effective solutions to safeguard both environmental and human health.

Bisphenol compounds (BPs) are among the most common phenolic substances in the environment, characterized by two phenol groups connected by an alkyl chain[29]. BPs are key components in polycarbonate plastics, widely used in consumer goods and containers for food and beverages[30]. Additionally, BPs are used in the production of epoxy resin linings in metal-based food and beverage cans, as well as in various other consumer products, such as thermal paper, medical devices, toys, electronics and water pipes[31,32]. Among these compounds, Bisphenol A (BPA) is the most prominent, being one of the most produced and consumed chemicals for manufacturing epoxy resins and

polycarbonate plastics[33]. The mass production and use of BPA have led to its widespread presence in the environment, where it has been detected in various media, including air, water, sediments, soil, aquatic organisms, and even human tissues[28,34].

BPs is also recognized as an endocrine-disrupting chemicals (EDCs). EDCs interfere with animal's and human endocrine system by mimicking or disrupting normal hormone functions, posing significant risks to both human health and wildlife[27,28]. In addition to acting through hormone receptors, EDCs can exert their effects through epigenetic mechanisms, altering the expression of key genes involved in reproduction and development[35]. BPs could interact with various physiological receptors, including estrogen receptors (α/β), estrogen-related receptor γ, androgen receptors, and thyroid hormone receptors. These interactions can have detrimental effects on reproductive organs, the nervous system, cardiovascular health, metabolic functions, and the immune system[34].

Due to BPA's numerous harmful physiological and behavioral health effects, many countries have banned its use in consumer products, especially in items like baby bottles. This has prompted the development of BPA alternatives in plastic production[36]. Bisphenol S (BPS), for instance, is commonly used as a substitute for BPA[30]. While these alternatives are believed to be more stable and less toxic, numerous studies suggest that they can still cause adverse effects in animals and humans, sometimes similar to or even worse than those caused by BPA[32,37].

Overall, BPs are prevalent worldwide and have been detected in various environments. BPs are linked to a range of health issues, including fertility problems, cancer, cardiovascular disease, obesity, allergic diseases, and neurodegenerative conditions such as Parkinson's and Alzheimer's diseases. Exposure to BPs can occur through both dietary and non-dietary sources, posing a significant threat to human health[38].

2. Sources of microplastics

Based on their origin, MP can be divided into primary and secondary types[39]. Primary MP, intentionally manufactured for commercial applications such as beads, pellets and nurdles used in industrial, synthetic textiles and personal care products are challenging to remove with conventional wastewater treatment technologies due to their small size. As a result, they are directly discharged into the natural environment[40]. Secondary MP are generated from the breakdown of larger plastic pieces through physical, chemical, and biological interactions, such as abrasion, photooxidation, weathering and microbial degradation[41,42].

Most plastics are initially used and discarded on land. Soils are considered the major storage for MP, which is closely related to the human daily activities including the large-scale use of agricultural mulch, organic manure application and uncontrolled waste dumps[20,43–45]. When MP are deposited in a terrestrial environment, some become fixed

in the soil. Over time, these MP may be vertically transported into deeper soil layers and/or eventually enter groundwater systems[46,47]. Another portion enters aquatic environment through various routes such as atmospheric deposition, surface runoff and sewage networks and eventually reaching the ocean via rivers, lakes and streams[41,48–50]. In addition, plastic waste from navigation, fisheries, and water operations is another source of MP pollution in water. MP pollution has also been widely observed in estuaries and inner bays, where it often originates from fiber films and debris generated by aquaculture activities[23,51–55].

In general, human activities, coupled with the rise in population, play a crucial role in the widespread distribution and increasing concentration of MP in various water bodies[24,56]. Also, driven by surface currents and wind forces, MP could migrate over long distances, which helps explain their ubiquitous presence in global waters[57].

2.1 Microplastics in freshwater

MP in riverine ecosystems have recently gained global attention due to their high concentrations found in sediments and water samples[58]. Generally, MP in rivers originate from land-based sources, specifically human-made sources resulting from industrial activities and high population densities[59]. Many studies have reported on the presence of MP in riverine ecosystems. For example, the Yangtze River in China produces the highest amount of plastic debris at 0.33 million tons per year, followed by the River Ganga in India at 0.12 million tons per year[60–62], and the Seine River, Paris[63], rivers in Switzerland[64], river Ganga, India[62], Rhine River, Germany[65] and Danube River, Austria[66].

As rivers are closely connected to freshwater and marine aquaculture environments, riverine ecosystems have been identified as areas where microplastics accumulate, primarily in the sediments. River ecosystems also serve as crucial pathways for transporting microplastics to marine environments[67,68].

2.2 Microplastics in marine systems

MP have been identified across the world's oceans, from nearshore to offshore and pelagic regions, on sea surfaces, deep sea, within water columns, and in seabed sediments[69–73]. They are ubiquitously distributed along the coastal and maritime zones of all continents, including the Arctic and Antarctica, affecting 42 countries' territories and 12 international sites[74]. MP loaded by rivers are significant sources of MP in marine environments. Furthermore, MP are more frequently found in the digestive tracts of fish captured near urban areas, likely due to the high input of microplastics from land-based sources[75]. It is estimated that the global release of MP into the oceans is approximately 1.5 million tons per year[76]. By 2025, it is projected that 250 million tons of plastics will

have accumulated in the oceans[77]. Studies have shown that MP are predominantly more abundant in marine environments than in freshwater environments[58].

The Yellow Sea and the Mediterranean Sea have been identified as hotspots for global marine microplastic contamination. Currently, 0.17% of the global ocean surface is at risk due to microplastic pollution, a figure that may rise to 0.52% by 2050 and 1.62% by 2100[78]. Microplastics have been reported in various locations worldwide, including the waters and sediments of Arctic ice[79], waters in and around China[51,80], the South Pacific subtropical gyre[81], the Atlantic Ocean[71], the Ross Sea of Antarctica[82], Lake Winnipeg in Canada[83], the Mediterranean Sea[84], the northwest Pacific[85], the Great Lakes tributaries of the USA[86], and the Thames River in the UK[87]. This widespread presence of MP reflects their uncontrolled distribution in international waters. Given that fish are a popular seafood consumed by humans, MP pollution in commercial sea fish has emerged as a significant concern for food security and human health[88].

3. Impact of microplastics on fish

The widespread presence of MP in global waters has raised concerns about their potential impact on aquatic life. In various water bodies worldwide, including the surface water of freshwater, the surface microlayer in seawater, the sediment layer in freshwater, and the bottom layer of seawater, almost all interlayers contain MP[89–94]. MP particles exhibit high bioavailability for different species, and the ingestion of MP has been observed in a variety of aquatic organisms, ranging from the smallest planktonic organisms to top predators, including micro-zooplankton, plankton, crustaceans, fish, sea turtles and large mammals at different levels[75,95–101] .

Aquatic plants and animals across different habitats inevitably interact with MP in multiple ways directly or in directly, including entanglement, ingestion, trophic transfer, bioaccumulation, and amplification[11,102]. MP are challenging for organisms to digest and decompose, which facilitates their spread throughout the global food chain. The higher an organism is to the top of the food chain, the more MP it tends to contain, posing a serious threat to human health[103–105]. MP-induced impairments in species range from minor disturbances in biological systems to severe adverse effects that can lead to mortality[106]. Both laboratory and field studies have demonstrated the negative effects of MP exposure on aquatic organisms.

3.1 Physical impacts

MP consumption could result in physical effects, including mechanical injury, reduced feeding and interrupted digestion[77]. After consumption, MP could accumulate in the gastrointestinal tracts of fish, causing physical blockages throughout the digestive system[107–109]. Such blockage could reduce feeding activity due to a false sense of satiation, could also cause internal damage, including structural and functional

deterioration, intestinal perforation and ulcerative lesions[106,110], which in turn would affect their growth rates, nutritional deficiencies and mortality rate in fish[111–113]. MP retention in the gill could also causes stress to the fish, reduced gill functioning. If MP particles become trapped in the gill lamellae, they would decrease the gill's effective surface area and respiratory efficiency, potentially leading to hypoxia[114]. In addition to damaging gill filament, MP could also increase the risk of gill infection[112]. Furthermore, the non-digestibility of MP increases the burden on the digestive system of fish, leading to severe intestinal lesions, hyperemia, digestive enzyme activity decreased and increased levels of trypsin and chymotrypsin[115].

In addition, MP exposure could also impair the feeding, weakened predatory performance, mating and swimming[116–118]. In a study involving Jacopever (*Sebastes schlegelii*), it was reported that exposure to MP resulted in a 65.4% decrease in weight gain rate, a 65.9% reduction in specific growth rate, and a 9.5% decrease in gross energy, compared to the control group[116]. In juveniles of Japanese rice fish (*Oryzias latipes*), Sub-lethal effects have been observed when they were fed with feed containing MP[119]. Feeding microbeads to the larvae and juveniles of holothuroids, asteroids fish, and echinoids resulted in delayed metamorphosis, altered body size, and changes in feeding rates, respectively[120–122]. In addition to physical impacts, the ingestion of MP can also result in chemical effects[123].

3.2 Biological impacts

The biological impacts of MP have been studied across various levels, the harmful effects include the oxidative stress, immune responses, neurotoxicity, growth, reproductive failure and metabolism alternations[24,124–128].

3.2.1 Oxidative stress

Among the molecular and cellular effects associated with MP, oxidative stress is a key mechanism driving biological responses[129,130]. In fish, oxidative stress can be triggered by various factors, including exposure to pollutants, pathogens, and environmental stressors[131]. MP, with their high surface area, could induce oxidative stress by releasing oxidizing species adsorbed on their surface or generating reactive oxygen species (ROS) during inflammatory reactions[15]. When fish are exposed to various MP, this disrupts the balance between ROS production and antioxidant capacity, leading to oxidative damage[132]. An increase in ROS serves as a key indicator of toxic reactions, leading to oxidative stress due to the imbalance between ROS generation and antioxidant capacity[132]. ROS are usually produced by mitochondria, and mitochondrial damage triggers the production of ROS[133].

Yang et al. (2020) reported that exposure to low concentrations of MPs caused the overproduction of superoxide dismutase (SOD) activity in *C. auratus* larvae[133]. Liu et al. (2019) found that MP induce oxidative stress by increasing ROS generation in

common carp[24]. An increasing in SOD activity following exposure to MP, leading to oxidative stress in *O. niloticus*[134]. Juvenile *Eriocheir sinensis* ingested MP particles could accumulate in the tissue and induce oxidative stress in the hepatopancreas[130]. Specifically, excessive production of ROS and alterations could also lead to DNA (deoxyribonucleic acid) damage[135]. In addition to causing oxidative stress in fish, MP could lead to increased activity of catalase and superoxide dismutase enzymes[136]. Therefore, in fish cells, the toxicity of MP primarily stems from oxidative stress, leading to disruptions in redox balance, damage to cellular components, and an overproduction of reactive oxygen species[137,138].

3.2.2 Immunotoxicity

MP are recognized as foreign substances and could impact fish immunity by either stimulating immune responses or suppressing immune function through immunotoxic effects. This indicates that MP could influence fish immune systems through various mechanisms[132]. Some scholars suggest that the accumulation of MP in fish tissues may disrupt the immune system by physically blocking nutrient absorption and causing chemical toxicity[139]. Other studies believed that MP exposure can affect the immune system of fish through the regulation of neutrophils extracellular trap release and granulocytes cells[132,140,141]. Meanwhile, effect studies indicate that once absorbed into the fish body, MP may interact with intestinal tissues and enter the circulatory system, leading to disruptions in the regulation of the immune response[142].

Moreover, histological examinations of exposed fish revealed that MP can trigger a significant inflammatory response in the target tissues[143]. Hirt and Body-Malapel (2020) and Bhagat et al. (2020) also reported that MP absorption in fish can disrupt the immune response by disturbing the oxidative and inflammatory balance in the intestines and interfering with the cytokine expression[142,144]. Inflammation is generally a protective response of tissues to harmful stimuli, usually injury, infection, or chemical irritants. In most cases, this process aids the immune system and its ability to clear the irritant[145].

In invertebrates, the innate immune system is the sole defense mechanism against pathogens, while in fish, it serves as a fundamental component of their defense system. For instance, the ingestion and accumulation of MP stimulate the inflammatory response and the innate immune system of fish[136,146]. Greven et al. (2016) and Espinosa et al. (2019) concluded that MP could disrupt the fish immune system by altering the organism's defense mechanisms and increasing the immunoglobulins[141,147]. Many studies have confirmed that a significant impact on the immune responses of fish after MP exposure, including elevated levels of lysozyme and neutrophils, apoptotic-like nuclear alterations and lysosomal membrane stability decreased[148–151].

3.2.3 Neurological damage

Bhagat et al. (2020) reported that exposure to MP in fish can result in the inhibition of several neurotransmitters, including dopamine, melatonin, gamma-aminobutyric acid,

vasopressin, oxytocin, serotonin, and kisspeptin[144]. Among these, acetylcholinesterase (AchE) is especially important as a sensitive biomarker of neurotoxicity in fish after exposure to MP, as it offers insights into potential damage to the neuromuscular cholinergic system[152,153]. AchE is crucial for maintaining appropriate neuromuscular function by inactivating acetylcholine, a process vital for cholinergic neurotransmission at both neuromuscular junctions and brain synapses[154,155]. There are some evidences that MP exposure could induce neurotoxicity by decreasing AchE and damaging lipid peroxidation in fish[132,134,152,154], leading to severe neurotransmission disorders, motor dysfunction, and behavioral abnormalities[133,151]. Several studies have documented that exposure to MPs inhibits AchE in various fish species, including Common carp, *Cyprinus carpio*[156]; juveniles of common goby, *Pomatoschistus microps*[157]; zebrafish larvae[151]; goldfish[133]; Amazonian cichlid, *Symphysodon aequifasciatus*[158] and African catfish[155,159].

3.2.4 Decreased reproductive system

Exposure to MPs can disrupt the endocrine system, posing serious implications for the endocrine health of fish[117,160]. MP can hinder reproductive functions in fish by competitively binding to receptors for sex steroid hormones[161]. This interference can lead to significant reproductive issues, including oxidative stress, apoptosis, and hormonal imbalances, which negatively impact fertilization, gonadal morphology, steroidogenesis, and the function of the hypothalamic-pituitary-gonadal (HPG) axis[162–165]. The HPG axis regulates reproductive functions by maintaining hormonal balance, and MP may disrupt this system, delaying ovarian development[166]. For instance, exposure of adult Japanese medaka (*Oryzias latipes*) to microfibers has been shown to alter HPG axis-related genes and promote vitellogenesis in males, indicating endocrine disruption and irregular maturation patterns[167]. MP exposure disrupts reproductive physiology by decreasing oocyte diameter and sperm mobility, reducing fertilization rates, affecting fecundity and offspring performance, while also lowering estradiol levels, which negatively impacts vitellogenesis, delays gonadal development, and reduces the gonadosomatic index[168–170]. Meanwhile, MP have been linked to diminished sperm quantity and quality, increased sperm DNA fragmentation[171,172], and altered reproductive behavior.

Furthermore, MP have potential toxicological effects on the embryos of aquatic organisms. Research indicates that MP can delay hatching in fish, which may subsequently impact predatory escape behavior and later stages of larval development[173]. For instance, early developmental exposure of zebrafish to MP resulted in a significant reduction in hatching rates due to teratogenic abnormalities in juvenile fish[174]. The toxic effects of microplastics can be transmitted to the next generation through germ cells, leading to intergenerational, multigenerational, or transgenerational reproductive effects on their offspring[168,175]. Several studies have demonstrated these transgenerational effects in fish, and underscore the idea that microplastic exposure in

adult fish can cause significant alterations in the physiological, morphological, and behavioral traits of their offspring[176–178].

3.2.5 Metabolic alterations

MP have extensive effects on fish metabolism, including lipid metabolism, oxidative stress, carbohydrate metabolism and toxin excretion[16]. The uptake of MP particles in fish can lead to liver poisoning, inflammation, and lipid accumulation in the liver[136]. Ingested MP can alter the metabolic process in fish by affecting cholesterol and triglyceride levels in blood serum, as well as regulating cholesterol levels in the liver. This can induce metabolic changes, such as the upregulation of lipids and the downregulation of amino acids[179].

Chronic exposure to MP can result in biosynthesis and metabolic disorders of lipids and lipoproteins in the fish liver, contributing to increased cholesterol and triglyceride levels. Additionally, exposure to microplastics can damage fish cell membranes, alter trans-membrane gradients, induce or inhibit enzymes associated with lipid metabolism, and change hormone levels related to lipid metabolism, leading to alterations in triglyceride and cholesterol levels[156]. The ingestion of MP can disrupt fish metabolism by altering the balance of triglycerides and cholesterol in the blood, as well as modifying the distribution of cholesterol in muscle and liver tissues[113]. For instance, exposure to MP has been shown to significantly increase blood glucose levels in fish, such as *Danio rerio*, due to impaired glucose metabolism in the liver[132]. Similarly, research by Banaee et al. (2019) found that exposure to MP led to a significant increase in glucose, cholesterol, and total protein levels in *Cyprinus carpio*[156]. The elevated glucose levels were linked to plasticizers affecting insulin resistance and disrupting glucose metabolism. Furthermore, metabolomic analysis also demonstrated that MP exposure could cause alterations in the liver's metabolic profile, interfering with lipid breakdown and energy generation pathways[136].

3.2.6 Impact on gut microbia

Gut microbiota plays a central role in numerous physiological, biochemical, and metabolic functions, including energy metabolism, nutrient absorption, and the synthesis of vital nutrients that contribute to host health[180,181]. Due to the critical roles of gut flora, an imbalance or dysbiosis in microbiota composition can increase susceptibility to pathogens, lead to metabolic diseases, and reduce host fitness[182,183]. Gut microbiome has also been shown to influence neural development and immune function through the "gut–brain axis" and "gut–liver axis"[184–186].

In recent years, several studies have demonstrated that MP can cause intestinal damage, penetrating the intestinal tissue of animals[187,188]. Experimental studies have shown that MP could alter the gut microbiota of zebrafish, leading to gut inflammation in adult fish and metabolic disorders in larval fish[124,189]. Additionally, MP fibers have been found to induce specific bacterial alterations in the gut, the gut-liver axis is often considered a

key pathway by which the gut microbiota affects the host's physiological responses[190,191]. The sensitivity of gut microbiota has made it a new toxicological target for environmental contaminants, as growing evidence suggests.

To date, zebrafish[192,193], marine medaka[178,194], crucian[195] and European sea bass[196] have been used as a model organism, demonstrating that exposure to MP can cause intestinal damage and alter the gut microbiome (Table 2-1).

Table 2-1. Overview of studies investigating the impact of MP on fish intestinal microbiome

Fish species	Microplastic type	Dose	Effect on microbiota	References
Zebrafish	Polystyrene (PS)	0.5μg/L and 5μg/L PS	*Proteobacteria* increased, and *Fusobacteriota* decreased	[192]
Zebrafish	Polystyrene nanoplastics (PS-NP)	0, 1, 10, and 100μg/L PS-NP	Phylum level: *Bacteroidetes* and *Firmicutes* decreased; *Fusobacteria* and *Proteobacteria* increased. Genus level: *Cetobacterium*, *Aeromonas*, and *Rhodobacter* increased.	[193]
Zebrafish	Polystyrene (PS)	1g/L PS	*Proteobacteria* decreased and *Actinobacteriota* increased	[197]
Marine medaka (*Oryzias melastigma*)	Polystyrene (PS)	5mg/g PS	In female: *Proteobacteria* decreased; In male: *Proteobacteria* and *Firmicutes* decreased, *Verrucomicrobia* increased	[194]
Crucian (*Carassius carassius*)	Polyethylene microplastics (PE-MP)	Intake 6.38, 12.18, and 22.33mg MP/fish/day	Phylum level: *Firmicutes* increased, *Fusobacteria* and *Bacteroidetes* decreased; Genus level: *Staphylococcus* and *Ralstonia* increased, *Bacteroides* and *Cetobacterium* decreased.	[195]
European sea bass (*Dicentrarchus labrax*)	Polypropylene (PP)	Add 10% (w/w) PP to fish diet	*Firmicutes* decreased and *Proteobacteria* increased	[196]
Zebrafish	Polystyrene (PS)	3mg/L 150μm PS	Phylum level: *Proteobacteria* and *Bacteroidetes* increased; *Fusobacteria* decreased; Genus	[198]

			level: *Cetobacterium*, *Acinetobacter* and *Kosakonia* decreased, *Aeromonas* and *Serratia* increased	
Marine medaka (*Oryzias melastigma*)	Polystyrene (PS)	2.5μg/mL 50nm and 45μm PS	Phylum level: *Bacteroidetes* decreased; Genus level: *Vicingus* and *Shewanella* decreased; *Lewinella*, *Pseudomonas*, *Thalassospira* and *Parahaliea* increased	[178]
Marine medaka (*Oryzias melastigma*)	Fluorescent polystyrene	100μg/L 2.5μm fluorescent polystyrene	Phylum level: *Proteobacteria* increased, *Firmicutes* and *Bacteroidetes* decreased; Genus level: *Ruegeria* increased,	[199]
Yellow croaker (*Larimichthys crocea*)	Polystyrene (PS)	$5.50*10^{-12}$ mg/L, $5.50*10^{-9}$ mg/L, $5.50*10^{-7}$ mg/L	*Bacteroidetes* and *Firmicutes* increased, *Proteobacteria* decreased	[200]
Juvenile guppy (*Poecilia reticulata*)	Polystyrene (PS)	100 and 1000μg/L PS	*Proteobacteria* increased and *Actinobacteria* decreased	[201]
Zebrafish	Polystyrene (PS)	50μg/L and 500μg/L PS	Phylum level: *Proteobacteria* decreased and *Fusobacteria* increased	[128]
Zebrafish	Polystyrene (PS)	100μg/L and 1000μg/L PS	*Bacteroidetes* and *Proteobacteria* decreased, *Firmicutes* increased	[189]
Zebrafish	Polystyrene (PS)	100 and 1000μg/L, 0.5 and 50μm PS	Phylum level: *Bacteroidetes* and *Proteobacteria* decreased, *Firmicutes* increased; Genus level: *Flavobacterium*, *Bacteroides*, *Rhodobacter*, *Stenotrophomonas*,	[181]

			Ralstonia, *Vogesella*, and *Plesiomonas* increased	

However, despite differences in the exposure concentration and fish species, these studies showed commonalities in terms of PS toxicity and sensitive microbial taxa. In general, *Proteobacteria*, *Bacteroidetes*, *Fusobacteria* and *Firmicutes* were the main bacteria in the healthy zebrafish and crucians of the control group at the phylum level[189,192,193,195,198]. The results showed that *Proteobacteria*, *Firmicutes* and *Bacteroidetes* are three-major bacterial in marine medak[178,194,199]. The gut microbiota in European sea bass were dominated by *Firmicutes* and *Proteobacteria*[196] but the dominating ones were *Proteobacteria* and *Actinobacteria* in Juvenile guppy[201]. The changes in microbial composition in *Proteobacteria* and *Fusobacteriota* may be related to the occurrence of inflammation and immune disorders[194,202]. *Firmicutes* are generally considered beneficial, playing a crucial role in nutrient absorption in fish guts and contributing to the host's anti-inflammatory defense[199]. The abundance changes of *Bacteroidetes* suggesting a potential risk of metabolic disorders and inflammatory response increased[178,181].

These studies have demonstrated that changes in the composition or abundance of microbiota could potentially lead to inflammation, immune system disorders, metabolic disruptions and cancer[203]. The microbiota is closely related to individual's health, and disruptions in microbial community networks or gut dysbiosis can increase the risk of disease and lead to early mortality.

4. Classification and selection of microplastics

MP are subject to intercompartmental transfer across terrestrial, aquatic, and atmospheric environments, and have been detected in all ecosystems on Earth. The current systematic classification of MP is predominantly based on their characteristics, including shape, size and chemical composition[204].

Various sources and weathering processes contribute to the formation of diverse MP shapes, including fragments, fibers, pellets, spheres, films, and foams[205,206]. The primary criterion for categorizing plastic debris is its chemical composition, which plays a crucial role in determining the degradation dynamics, environmental distribution, and ultimate fate of MP[204]. At present, the chemical composition of MP mainly includes polyethylene (PE), polystyrene (PS), polyethylene tetraacetate (PET), polyvinyl chloride (PVC) and polypropylene (PP)[204,207].

Polyethylene (PE), the most extensively utilized plastic polymer, is a high-molecular-weight synthetic polymer composed of a linear saturated hydrocarbon structure, represented by the repeating unit -$[CH_2\text{-}CH_2]_n$-[208,209]. PE constitutes approximately 30%

of the global demand for plastic polymers, with an estimated annual production of 140 million tons[210]. Furthermore, PE is non-biodegradable, it exhibits prolonged persistence in both aquatic and terrestrial environments, accumulating in water bodies and soil over extended periods[211]. Given its enormous production and relatively low recycling rate, polyethylene plastic waste has emerged as a significant environmental concern, and posing significant negative effects on terrestrial and aquatic organisms[212]. Therefore, in our present study, we selected polyethylene as our research target to discuss its effects on aquatic organisms.

5. Bisphenol compounds in aquatic environment

Bisphenol compounds (BPs) are synthetic compounds that do not occur naturally but have become pervasive in the environment due to their high production and extensive use[213]. BPs commonly used in numerous consumer products, are introduced into aquatic ecosystems through various pathways, such as atmospheric deposition, inefficient wastewater treatments, urban sewage landfill leachates, and domestic waste[28,214,215].

Human activities are the sole source of BPs in the environment, with polycarbonate plastics and epoxy resins being key contributors to BPs contamination in water[216]. BPA has been detected worldwide, in both seawater and sea sand[217], and is the most frequently found BPs in river, lake, and seawater, with concentrations ranging from 0.00001 to 85.5μg/L[218,219]. Studies have reported BPA concentrations of up to 517ng/L in the Sinos River basin in Brazil[220], 217ng/L in China's Taihu Lake[221], and 31.45ng/L in the Pearl River in southern China[222].

In addition to BPA, other bisphenol substitutes like BPS have been found in high concentrations in various water bodies. For instance, BPS concentrations reached 65,600ng/L in the Liuxi River, China[223], and 7,200ng/L in surface water from the Adyar River, India[224]. BPS has also been detected in the Liaohe River (14ng/L), Taihu Lake (6ng/L), and the Hunhe River (11ng/L) in China[225].

Furthermore, BPs have even been found in both source water and drinking water in China[226,227]. Overall, the increasing presence of BPs in aquatic environment, despite the restrictions on BPs in consumer products, highlights the ongoing environmental threat posed by these chemicals.

6. Toxic effect of bisphenol compounds

The presence of BPs in aquatic environments can significantly impact not only the biodiversity and productivity of phytoplankton communities but also exert severe adverse effects on organisms[228]. These compounds have the potential to disrupt the functioning of entire aquatic ecosystems[229].

Numerous studies have demonstrated that fish exposed to BPs experience a range of harmful effects. These include oxidative stress[230], disruptions to the endocrine system and reproductive processes[231–233], and adverse impacts on spermatogenesis[234]. Additional observed effects include developmental malformations and feminization[235], neurotoxicity[215,236], hematopoietic abnormalities[237], transcriptional alterations[238], behavioral changes[239], and lipid metabolism disorders[240].

6.1 Effect on endocrine disruption/ reproduction

The endocrine system in zebrafish is regulated by the interactions of endocrine hormones along the hypothalamic-pituitary-thyroid (HPT) axis and the hypothalamic-pituitary-gonad (HPG) axis. BPs can interfere with these processes by affecting the expression of related genes and altering hormone and enzyme levels, ultimately disrupting the endocrine system and impairing growth in fish[235,241].

Reproduction in fish is controlled by a conserved endocrine pathway involving several tissues, including the hypothalamus, pituitary gland, liver, and gonads. This system is regulated by various hormones such as gonadotropins, steroids, and vitellogenin, along with receptors like estrogen receptors (ERs) and androgen receptors (ARs), which are part of the estrogenic signaling pathway[242]. BPs disrupt normal endocrine function by binding to these receptors, mimicking or blocking natural hormones.

BPs has been shown to disrupt reproductive and thyroid endocrine systems in zebrafish by altering reproductive genes and hormone levels. For instance, BPA exposure induces vitellogenin production in male zebrafish, a marker of estrogen interference, leading to disrupted thyroid hormone regulation[34]. Studies have also shown that BPA exposure reduces hatchability, decreases thyroid hormone concentrations, and increases thyroid-stimulating hormone levels in zebrafish[243]. Studies have also found BPs can impair fish reproduction by affecting gonadal development and gamete quality. In species such as common carp (*Cyprinus carpio*), zebrafish, and *Catla catla*, BPs has been found to increase oocyte atresia and vitellogenic follicles[244,245]. Additionally, high concentrations of BPA (10μM) have been shown to skew sex ratios, significantly increasing the female population in zebrafish[246].

Importantly, the effect of BPs are not limited to exposed individuals but also induce transgenerational reproductive impairments, such as increased embryo mortality and reproductive dysfunction in subsequent generations[36]. Additional studies have documented that parental BPs exposure has been associated with higher risk of offspring mortality, congenital malformations, reduced swimming speed, cardiac arrest and increased apoptotic activity[247–250].

6.2 Effect on oxidative stress

Free radicals, such as reactive oxygen species (ROS), are harmful byproducts of various biological processes. Normally, these free radicals are neutralized by the body's antioxidant defense mechanisms[251]. Oxidative stress occurs when there is an imbalance between the production of ROS and the biological system's ability to detoxify them or repair the resulting damage[252].

BPs has been shown to disrupt mitochondrial oxidative balance by either increasing ROS production or reducing the antioxidant defense capacity[252]. This BPs-induced increase in ROS has been documented in various organs and cell types in both in vivo and in vitro zebrafish experiments, with exposure concentrations ranging from 0.1 to 1000μg/L[253]. When ROS generated from BPs metabolism exceed the capacity of the antioxidant system, it can overwhelm the cell's defenses, leading to oxidative damage[242].

In addition, exposure to BPs has been linked to oxidative stress responses, including reduced superoxide dismutase (SOD) activity and increased levels of malondialdehyde (MDA), a marker of lipid peroxidation[236]. Studies using BPA concentrations ranging from nanomolar to micromolar levels have consistently shown a decrease in the activity of key antioxidant enzymes such as SOD, glutathione peroxidase (GPx), and catalase (CAT) in various tissues, including the brain, hepatopancreas, intestine, gonads, and whole embryos of zebrafish[253–256].

A significant consequence of oxidative stress is its potential to impair metabolic performance, which has broad ecological implications. For example, Wu and Seebacher (2021) reported that BPs exposure reduces oxidative metabolic capacity and increases reliance on anaerobic metabolism, particularly in warm climate environments[257]. This shift in metabolism negatively affects the growth and activity of zebrafish[258], highlighting the ecological risks posed by BPA and its analogs.

6.3 Effect on growth and development

While BPs is primarily known for its adverse effects on reproduction, it also negatively impacts the growth and development of fish embryos, larvae and juveniles. Exposure to BPA and its analogs can disrupt normal developmental processes, leading to various negative outcomes in fish.

One significant effect of BPs is on the timing of zebrafish hatching. Delays or alterations in hatch timing can reduce offspring survival rates. Several studies have reported significant hatching delays in zebrafish embryos exposed to BPs[235,259]. However, some research has found the opposite effect, with accelerated hatching rates following BPs exposure. For example, Qiu et al. (2018) observed an increased hatch rate in zebrafish exposed to BPs compared to a control group[260]. This accelerated

hatching could be a stress response, where embryos attempt to escape a highly stressful environment[258].

In addition to hatching disruptions, BPs have been linked to deformities and developmental abnormalities in zebrafish. Prolonged exposure to BPs can lead to early mortality, with embryos and larvae dying after 120 hours of treatment, and significantly reduced offspring survival in adults exposed to BPs[261,262]. Developmental malformations, such as cardiac edema, spinal malformations, and craniofacial deformities, have also been observed in zebrafish larvae following BPs exposure[259]. These deformities extend to heart and vascular development, with recent studies highlighting that the cardiovascular system is particularly susceptible to BPs-induced toxicity[263].

BPs have all been associated with developmental deformities in zebrafish[259]. These findings suggest that bisphenols disrupt various developmental processes, leading to direct developmental abnormalities as well as stunted growth[264]. Overall, the effects of BPs on growth and development pose a serious risk to fish populations, with potential long-term ecological consequences.

6.4 Effect on neurotoxicity

Recent studies have identified BPs, particularly BPA, as neurotoxicants in zebrafish[265,266]. BPs have been shown to disrupt the central nervous system (CNS), potentially leading to impaired endocrine function and altered behaviors[258]. The hypothalamic-pituitary-hormonal axis has emerged as a significant target for BPs, with evidence suggesting that exposure to BPs affects neurogenesis and motor neuron function[267].

Low-dose exposure to BPA during CNS development in zebrafish has been associated with hyperactivity in larvae and learning deficits in adult fish[268]. Similarly, BPA and its analog BPS have been found to induce precocious neurogenesis and hyperactive behaviors in zebrafish larvae[269]. Furthermore, exposure to BPS altered retinal function in male zebrafish, impairing vision and locomotor behavior, and down-regulating key genes required for normal neural development[270,271]. These studies highlight the neurotoxic effects of BPs, particularly in affecting sensory and motor systems.

A recent study demonstrated that BPs could cross the blood-brain barrier in zebrafish larvae, disrupting dopaminergic and cholinergic pathways. This led to altered behaviors, such as changes in color preference, decreased movement, and reduced distance traveled[239]. Such findings suggest that BPs could cause significant neurological damage, impairing motor functions and behaviors in aquatic organisms, similar to the effects observed in mammals and humans. The ability of BPs to penetrate the blood-brain barrier and disrupt neurotransmitter systems is likely a key mechanism behind these neurotoxic effects, contributing to hyperactivity and altered behavioral patterns[36].

Additionally, emerging research is exploring the role of BPs in shaping the gastrointestinal microbiome of zebrafish[272]. This area of study is particularly relevant to neurotoxicity due to the strong connection between the gut and brain, known as the gut-brain axis[258]. Understanding this relationship may further elucidate the broader neurotoxic impacts of bisphenols on aquatic species

6.5 Effect on metabolism

The liver, a central organ for metabolizing toxic substances, is particularly susceptible to damage from exogenous chemicals. Hepatotoxic effects of BPs have been extensively studied, particularly in mammals[273]. Studies have shown that BPs induce lipid accumulation in the liver of mice, primarily by regulating sterol regulatory element binding proteins[274]. BPs exposure in animal models disturbs redox balance, induces oxidative stress, endoplasmic reticulum stress (ERS), apoptosis, mitochondrial dysfunction and inflammation in the liver[275,276].

In aquatic species, similar hepatotoxic effects have been observed long-term exposure to BPs in common carp (*Cyprinus carpio*) increased hepatic somatic index, causing oxidative stress and immune disturbances[277]. Additionally, BPs disrupted metabolic homeostasis and triggered inflammatory responses in the liver of Labeo bata (*Cyprinidae, Cypriniformes*)[278]. Notably, the upregulation of *fatp1*, a key gene involved in the transport of long-chain fatty acids, was observed in common carp liver following BPs exposure. This overexpression leads to enhanced fatty acid uptake, resulting in lipid accumulation within liver tissues[273].

In zebrafish, exposure to BPs at lower concentrations (5μg/L) increased triglyceride storage and promoted fatty acid synthesis, while higher concentrations (20μg/L) stimulated de novo lipogenesis and cholesterol accumulation in adult females[279]. Similar findings were observed in adult male zebrafish, where BPs exposure led to the upregulation of genes associated with lipogenesis[280].

In addition to lipid metabolism, BPs also affect glucose homeostasis. Studies in zebrafish have shown that BPs exposure leads to insulin resistance and disrupted glucose regulation[281]. BPA exposure increased body weight, hepatic triglyceride levels, and lipid accumulation in male zebrafish[280]. Furthermore, BPA significantly raised fasting blood glucose levels and body weight compared to controls[254]. These disruptions in glucose homeostasis, characterized by elevated fasting glucose and reduced insulin levels, suggest that BPs may contribute to the development of obesity in zebrafish[282].

7. Bisphenol compounds alternatives

Due to the potential health risks that BPA poses to humans and other organisms, some countries and regions have implemented restrictions on its industrial use[283]. In response to both proposed and existing regulatory measures, manufacturers of BPA-containing products have sought alternative substances that offer similar functionality but are presumed to be less harmful[284].

As a result, various BPA substitutes have been introduced to the market, including bisphenol F (BPF) and bisphenol S (BPS), along with other alternatives such as bisphenol Z (BPZ), bisphenol E (BPE), bisphenol S-MAE (BPS-MAE), bisphenol P (BPP), bisphenol AP (BPAP), bisphenol B (BPB), bisphenol C (BPC), bisphenol AF (BPAF), and others[284,285].

While significant attention has been given to BPA substitutes in Europe and the United States, a global perspective, particularly on the regulatory framework and usage patterns in other regions such as China, remains underexplored. In China, BPA and its substitutes are extensively used in various industrial applications, including plastic production, thermal paper, and epoxy resins[286,287]. Although China has implemented restrictions on BPA in specific products, such as infant bottles and food contact materials[287], there is currently no comprehensive regulatory framework governing BPA alternatives like BPS and BPF.

BPS and BPF are two well-known BPA substitutes, they have widespread consumer and commercial use[231,288]. However, similar to BPA, BPS and BPF have been widely detected in environmental and human samples, including sediment, indoor dust, food, consumer products, urine, and serum, indicating their extensive use and potential exposure risks[32,231,289]. Among them, BPS has a chemical structure highly similar to that of BPA, but it contains a sulfone group with strong electron-withdrawing properties, along with two hydroxyl groups. These structural characteristics make BPS more acidic and chemically stable compared to other bisphenols, including BPA[290]. For instance, BPS exhibits greater resistance to heat and sunlight than BPA. Studies have shown that the biodegradability of bisphenol analogues in seawater follows the order: BPF > BPA > BPS[291,292]. BPA and BPS are the most commonly detected bisphenol compounds in biological samples, and BPS is also detected at higher concentrations in human urine and environmental samples[293,294], highlighting its widespread exposure and environmental persistence. Therefore, BPS has become one of the primary substitutes for BPA and is widely present in consumer products, leading to extensive human exposure. Given its prevalence, we have selected BPA and BPS as the focus of this study to investigate its impact on aquatic organisms in the environment.

8. The relationship of BPs and MP in aquatic environment

Recent studies have highlighted the potential environmental and human health risks associated with MP[295]. MP can interact with other pollutants, including antibiotics[296],

organic chemicals[297,298], heavy metals[299–301], and endocrine-disrupting chemicals such as BPs[302], potentially influencing their bioavailability and toxicity, which underscores the need to examine the combined ecotoxicological effects of MP and chemical contaminants. Due to their large surface area and hydrophobicity, MP can absorb various pollutants, increasing the bioavailability of these contaminants to aquatic organisms through the "Trojan horse" effect[303]. This characteristic allows MP to act as carriers for pollutants, such as BPs, contributing to increased environmental risks[304,305].

MP have been found to serve as both sinks and sources of BPs in aquatic systems[306]. Several studies have shown that MP can adsorb BPs through hydrogen bonding and hydrophobic interactions, thus concentrating BPs on their surfaces[307,308]. Fish exposed to MP have been shown to contain significantly higher concentrations of BPs, suggesting a direct relationship between MP presence and BPs contamination in aquatic life[30].

Moreover, MP can enhance the bioaccumulation and toxicity of BPs in aquatic organisms. Co-exposure to MP and BPs has been found to be more toxic to aquatic species than individual exposures to either pollutant. For instance, studies demonstrated that combined exposure to MP and BPA significantly increased toxicity in blood clams and impaired gonadal development in whiteleg shrimp[160,309]. In zebrafish larvae, co-exposure to BPs and MP increased lethality and amplified toxic effects[310]. This synergistic toxicity could be due to shared toxic targets, facilitated internalization of BPs in the presence of MP, or disruption of BPs detoxification mechanisms by MP[308,311,312].

Overall, co-exposure to MP and BPs alters their toxicity in aquatic organisms compared to single exposures. This highlights the urgent need to further investigate the combined impacts of these two pollutants to better understand their ecological and health risks[308,313].

7. Conclusions

The coexistence of microplastics (MP) and bisphenol compounds (BPs) in aquatic ecosystems presents a significant and multifaceted threat to both wildlife and human health. Individually, these pollutants cause considerable harm, MP physically damage aquatic organisms, disrupt digestion, induce oxidative stress, and impair reproductive and immune functions, while BPs act as endocrine disruptors, leading to hormonal imbalances and reproductive issues. When combined, the effects of MP and BPs are exacerbated, as MP serve as carriers that enhance the bioavailability of bisphenols, amplifying their toxic effects. This synergistic toxicity leads to more severe oxidative stress, neurotoxicity, reproductive failure, and developmental abnormalities in aquatic species, particularly fish.

Importantly, these pollutants do not only remain within aquatic ecosystems but can enter the human food chain through the consumption of contaminated seafood, posing direct risks to human health, including endocrine disruption, immune system impairment, and potential long-term reproductive effects. Therefore, the co-presence of MP and BPs in the environment heightens ecological and public health risks, underscoring the urgent need for comprehensive pollution control measures and further research to fully understand their combined impacts. Addressing this dual threat is critical to safeguarding both environmental integrity and human well-being.

Chapter 3

Determination of bisphenol compounds and the bioaccumulation after co-exposure with polyethylene microplastics in zebrafish

Adapted from:
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Abstract

Knowledge regarding the combined toxicity mechanism of bisphenol compounds and microplastics (MP) on organisms remains limited. In this study, we first developed an accurate and sensitive method to simultaneously quantify two bisphenol compounds and evaluate their accumulation and tissue distribution after co-exposure with MP in zebrafish. Then, we determined the bioaccumulation potential of bisphenol A (BPA) and bisphenol S (BPS) in adult zebrafish in the absence and presence of MP. Bisphenol compounds were found to accumulate in different tissues of zebrafish, with BPS showing lower accumulation levels compared to BPA. Importantly, we discovered that the presence of MP could exacerbate the accumulation of bisphenol compounds in biological tissues. These findings highlight the enhanced bioavailability and risk posed by the co-exposure of bisphenol compounds and MPs, underscoring the need for further investigation into their combined environmental and biological health impacts.

Keywords: bisphenol compounds; microplastic; co-exposure; bioaccumulation

1. Introduction

The endocrine-disrupting chemical bisphenol A (BPA) is nearly ubiquitous in natural environments[314]. In recent years, growing evidence have been confirmed that it could bind to various hormone receptors and subsequently affect normal physiological processes, such as reproductive disorder, immune responses and cardiovascular disease[315–317]. The adverse effects of BPA on aquatic organisms and ecosystem health have caused significant public concern[277,318]. For this reason, BPA has been banned in many products and have gradually begin to use its alternatives, such as bisphenol S (BPS)[231]. However, similar chemical structure and estrogenic activities may lead to similar physiological effects[319,320].

Microplastics (MP), another global environmental issue also severely threatens natural ecosystems and humanity[309]. Most importantly, MP has emerged as a global concern, not only because of their ecotoxicological impacts but also because of their interactions with other pollutants[321]. Many studies have proved that MP could interact with other pollutants owing to their strong hydrophobicity[312,322,323], causing further eco-toxicological impacts[324]. Furthermore, growing evidence suggested that MP could interact with bisphenol compounds in aquatic environments and accumulated in exposed organisms, causing further negative effects[303,304]. The toxic effect of co-exposure of bisphenol compounds and MP have been extensively studied, and it has been demonstrated that the presence of MP could affect the bioavailability and toxicity of bisphenol compounds to organisms[160,313,325]. However, to the best of our knowledge, whether the presence of MP could affect the accumulation and distribution of bisphenol compounds within organisms are rarely investigated. Thus, there is an urgent need to investigate the bioaccumulation of bisphenol compounds after co-exposure with MP in aquatic organisms.

At present, the determination methods for bisphenol compounds are mainly focused on biological human matrices[326–328], food products[329–331] and environmental media[332,333]. There are few methods to determine with a single analysis the concentrations of multiple bisphenol compounds in biological samples[334], and information on the bioaccumulation of bisphenol compounds in fish, especially at tissues level, is still limited. Because of the serious adverse effects of bisphenol compounds, it is necessary to develop a reliable and sensitive analytical method. Here, we developed an accurate and sensitive HPLC-MS/MS approach for the simultaneous measurements of BPA, and its alternatives BPS in six tissues from zebrafish, namely brain, gill, muscle, gonad, liver and intestine.

Therefore, to gain a better understanding of the fate and potential toxicities of bisphenol compounds and MP, the accumulation and tissue distribution of target chemicals, alone or in combination with MP were investigated in zebrafish. The results could not only

help researchers to understand uptake and distribution of bisphenol compounds within the presence of MP but also may provide deeper insight into their potential toxicity.

2. Materials and method

2.1. Chemicals

The standard of BPA (CAS:80-05-7) (purity 99.8%) and BPS (CAS:80-09-1) (purity 99%) were purchased from Aladdin Biochemical Technology Co., Ltd (Shanghai, China). Polyethylene microplastics with particle size of 25μm were purchased from Zhichuan Technology Co., LTD (Jiangsu, China). Methanol and acetonitrile were purchased from Thermo Fisher Scientific Inc (Shanghai, China). All reagents were HPLC grade. The stock solutions of BPs (400μg/L) were prepared by dissolving appropriate amount of each standard in methanol. All solutions were stored at -20°C until use.

Ammonium acetate, glacial acetic acid and ammonium hydroxide (analytical grade) were provided by Beijing Chemical Co. (Beijing, China). β-glucuronidase from *E.coli* K12 was supplied by Roche Diagnostics GmbH (Mannheim, Germany). Ultrapure water was purified through a Milli-Q plus system (Millipore, Bedford, MA, USA). The SPE C_{18} cartridges were purchased from Meizheng Bio-Tech Co., LTD (Shandong, China). 0.22μm Filter Unit was from Bonna-Agela Technologies Co.,Ltd, (Beijing, China).

2.2. Exposure experiment and sample collection

Adult zebrafish (AB-wild type, aged 5 months) were purchased from the aquarium department of Hongdagaofeng and have been continuously cultivated in the laboratory for two weeks before the exposure tests (14h light/10h dark cycle, 25.0 ± 1.0 °C). During the acclimation period, fish were fed two times daily and rearing water were renewed every three days.

Adult zebrafish were randomly selected and exposed to different treatments and control (Ctr) group (zebrafish were exposure to dechlorinated tap water), BPA group (100μg/L of BPA), BPS group (100μg/L of BPS), MP group (100μg/L of MP), MA group (100μg/L of BPA +100μg/L of MP) and MS group (100μg/L of BPS +100μg/L of MP). The concentration of exposure (100μg/L) was based on the environmentally relevant concentrations and could induce clearly effects and identify possible mechanisms of toxicity[335,336].

Six replicates were set for each treatment, each of which contained 4 L liquid and 10 adult fish in a 5 L glass beaker. The solutions were changed every 3 days to ensure that the concentration of the tested substance was stable. The exposure period was 35d.

During the experiment, external conditions, including temperature, humidity and light cycle, were consistent with the domestic environment.

After exposure of 35d, fish were starved for 24h, five zebrafish were collected randomly per replicate and anaesthetized in MS 222 (Tricaine, Sigma-Aldrich). The muscle, brain, gill, gonad, liver and intestinal tissues were removed quickly and set on ice. All samples were stored at −80 °C until extraction and analysis.

2.3. Sample preparation

The zebrafish tissue samples were ground by a homogenizer, added 1mL of 1mol/L ammonium acetate buffer solution (pH5.0, 7.71g ammonium acetate dissolved in 93.4mL of ultrapure water, 6mL Glacial acetic acid and 600μL of β-glucuronidase). After the mixed solution was hydrolyzed in a 37°C water bath for 12 hours, 1mL methanol and 1mL water were added to the samples, thoroughly mixed with a refrigerated grinder for 10 min, extracted ultrasonically for 30 minutes, and centrifugated for 10 min at 10,000 rpm. The liquid supernatants were transferred to new 50mL centrifuge tubes. The extraction described was repeated for two times.

Samples were further purified with solid phase extraction (SPE) on a C_{18} cartridge which was preconditioned successively with 10 mL of methanol and 10 mL of water. After sample uploaded, the cartridge was eluted with 10 mL of methanol/water (5:95, v/v) and the elutant was discarded. The samples were then eluted with 10 mL of 5% ammonium hydroxide and were brought to dryness under gentle flow of high purity nitrogen and reconstituted with 1 mL of methanol/water (1:1, v/v). The final solution were filtered through 0.22μm syringe filter then into individual 2 mL glass vial prior to instrumental analysis.

2.4. Analytical conditions

Chromatographic analysis were performed on an ExionLC AE system (AB SCIEX, Framingham, USA). A 22-minute gradient on a HSS T3 column (2.1 x 100 mm, 1.8μm) was employed for efficient separations. The composition of the mobile phase was acetonitrile (A) and 0.1% formic acid in water (B), with a flow of 0.3 mL/min and the following gradient: 0–1 min, 20% A; 1–13.5 min, 20–95% A; 13.5–18 min, 95% A; 18–22 min, 20% A. The injection volume was set to 2μL.

Mass spectrometry analysis was carried out on a SCIEX Triple Quad 4500 system with electrospray ionization (ESI) probe in negative mode. The MS source conditions were as follows: curtain gas (CUR), 20 psi; collision gas (CAD), medium; nebulizing gas (GS1), 50 psi; heater gas (GS2), 60 psi; ion spray (IS) voltage, 4500 V; source temperature, 500ºC.

2.5. Data analysis

Statistical analysis and data illustrated by GraphPad Prism 10.2.0. Significant differences between groups were tested by one-way analysis of variance (ANOVA).

3. Results

3.1. UPLC-MS/MS conditions

A BEH C_{18} column (100 mm × 2.1 mm, 1.7µm) and HSS T3 columns (2.1 x 100 mm, 1.8µm) were selected to the separation of the target compounds. However, it was found that BPS exhibited poor retention on C_{18} column, resulting in rapid elution. In contrast, T3 column provided better retention and separation for BPA and BPS, indicating a more suitable interaction with the stationary phase for the target compounds[337]. Then, the HSS T3 column was selected to analyze the targeted molecules. In preliminary experiments, methanol and acetonitrile at different concentrations were tested as the organic mobile phase. When acetonitrile was used as the organic phase, it had the better peak shape and peak broadening without obvious peak tailing. Furthermore, several studies have recommended that acidification of mobile phase could improve sensitivity and ionization efficiencies, and therefore 0.1% formic acid was added to the water phase[338,339]. The sensitivity of both target compounds was higher than that of the pure water phase. The UPLC-MS/MS chromatograms of BPA and BPS standards and their MS^2 spectra pattern were shown (Figure 3-1).

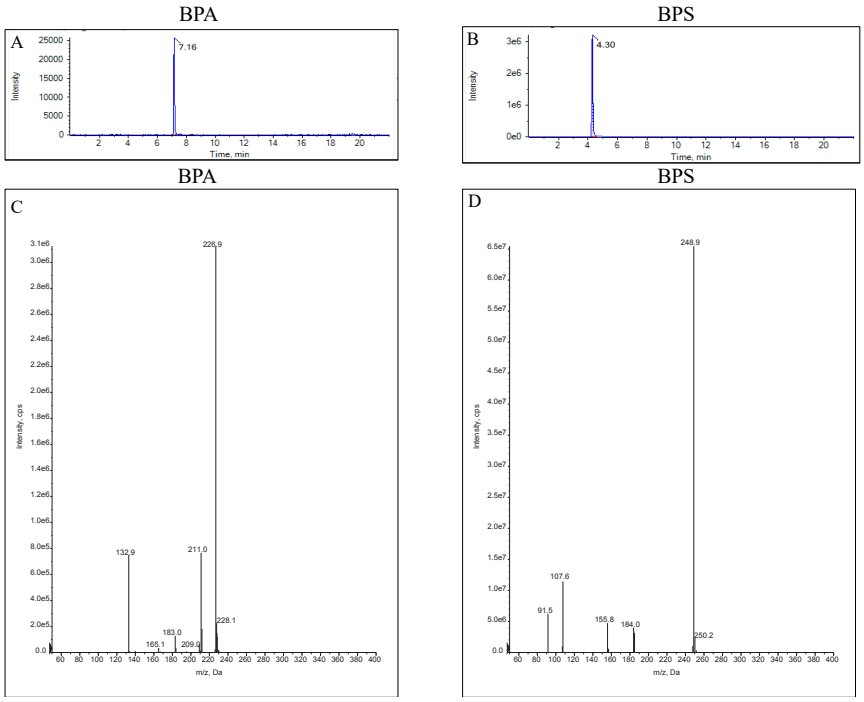

Figure 3-1. The UPLC-MS/MS chromatograms of BPA (A) and BPS (B) standards and their MS^2 spectra pattern (C: BPA; D: BPS).

3.2. Sample preparation

Regarding widely different physicochemical properties of two target compounds, the sample preparation is an important step to simultaneously extract all the analytes from complexity biological matrix. Acetonitrile, acidified acetonitrile (acetonitrile/formic acid=99:1, v/v), 75% acetonitrile, 75% acidified acetonitrile (75% acetonitrile/formic acid=99:1, v/v), ammonia acetonitrile (acetonitrile/ammonia solution=99:1, v/v), EDTA-Mcllvaine buffer solution (acetonitrile/ EDTA-Mcllvaine buffer solution=9:1, 8:2, 7:3, 6:4, 5:5) and ammonium acetate buffer solution (pH5.0) were tested to simultaneously extract BPA and BPS from zebrafish tissues. The recoveries of all these extract solvents were shown in Table 3-1. Therefore, ammonium acetate buffer solution (pH5.0) generated better recoveries than the other solvents and finally was selected as the extraction solvent for further study. Ammonium acetate can serve as a stabilizing background electrolyte and could effectively dissolve and extract bisphenol compounds, improving extraction efficiency and accuracy[340]. Next, the extraction effects with or without β-glucuronidase were also compared. It was found that the presence of β-glucuronidase significantly reduced background noise (Figure 3-2), possibly because the enzymatic reaction led to the degradation of complex matrix components and minimized matrix effects. Furthermore, previous studies have demonstrated that bisphenol compounds usually be conjugated forms in animal samples. Analytical methods for determining bisphenol compounds in biological samples usually using enzymatic hydrolysis to convert the conjugated into their free forms[341]. Therefore, β-glucuronidase was used in this study to ensure better extraction efficiency.

Table 3-1. The recoveries of BPA and BPS in all these extract solvents. (n=3).

	BPA	**BPS**
Extract solvents	Recoveries (%)	Recoveries (%)
Acetonitrile	72.6 ± 4.2	62.2 ± 15.6
Acidified acetonitrile	84.0 ± 1.9	66.0 ± 2.7
75% acetonitrile	68.3 ± 6.2	67.9 ± 5.8
75% acidified acetonitrile	69.7 ± 1.4	62.3 ± 2.9
Ammonia acetonitrile	75.7 ± 8.5	54.4 ± 16.6
Acetonitrile: EDTA-Mcllvaine=9:1	79.5 ± 6.8	62.7 ± 11.1
Acetonitrile: EDTA-Mcllvaine=8:2	75.8 ± 2.4	70.3 ± 3.2
Acetonitrile: EDTA-Mcllvaine=7:3	45.4 ± 3.1	39.2 ± 9.2
Acetonitrile: EDTA-Mcllvaine=6:4	34.5 ± 2.3	37.6 ± 3.0
Acetonitrile: EDTA-Mcllvaine=5:5	50.9 ± 10.1	54.2 ± 1.1
Ammonium acetate buffer solution	98.5 ± 4.5	92.7 ± 0.8

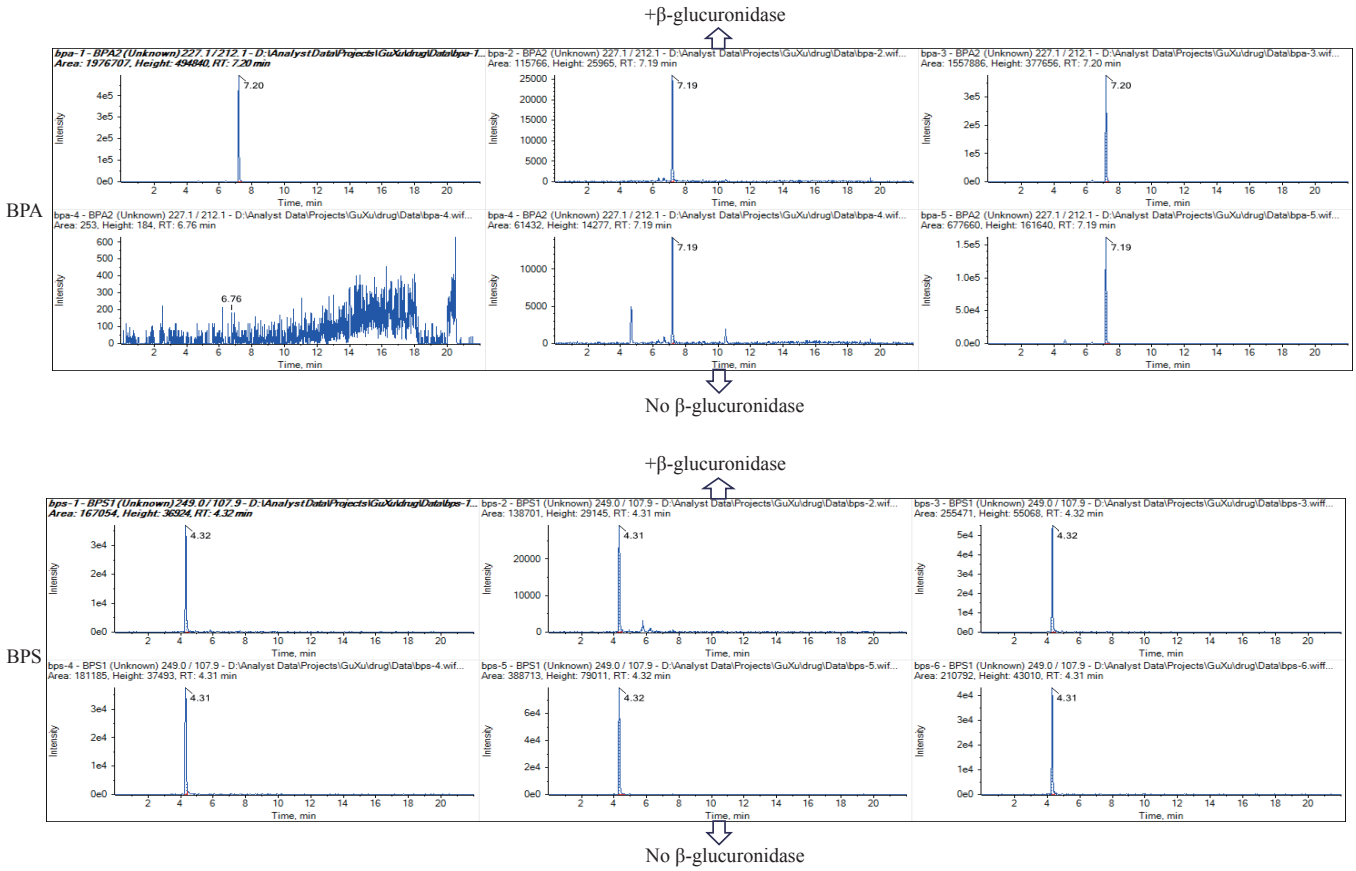

Figure 3-2. The chromatograms of extraction affect BPA and BPS with or without β-glucuronidase.

3.3. Method validation

Accuracy, precision, linearity, limit of detection (LOD), and limit of quantification (LOQ) were validated for the developed method. Accuracy and precision were expressed as recoveries and relative standard deviations (RSDs), the recovery assay was determined by six replicates at three different concentration levels (Table 3-2). Satisfactory recoveries for BPA from 78.8-109.5%, and 72.9-113.2% for BPS were obtained from different zebrafish tissues, with RSD<12%, indicating efficient extraction.

The linearity was studied using matrix-matched standard solutions in triplicate at eight concentration levels are summarized in Table 3-3. The correlation coefficients of the calibration curves were all higher than 0.99. The LOQ of the BPA and BPS ranged from 0.6-3.0μg/L and from 0.8-3.0μg/L, respectively, which indicated that proposed analytical method is highly sensitive. In this study, all of the matrix effects were also presented in Table 3-3. Different tissue samples showed different matrix-induced effect or matrix enhancement (-11.2-12.0% for BPA and -13.8-13.9% for BPS). The reason

for this effect may be complex composition and organic matter in biological samples, and some unknown compound influence ionization efficiency for these analytes[341,342].

Table 3-2. Spiked average recoveries and relative standard deviations (RSDs) of BPA and BPS in different zebrafish tissues. (n = 6).

Tissues	Spiked (μg/L)	BPA				BPS			
		Intra-day		Inter-day		Intra-day		Inter-day	
		Recovery (%)	RSD	Recovery (%)	RSD	Recovery (%)	RSD	Recovery (%)	RSD
Brain	5	78.8	8.2	89.2	8.7	81.6	3.6	72.9	11.1
	10	83.7	4.3	90.3	5.8	84.3	1.8	86.8	4.85
	20	83.9	5.5	91.2	3.9	79.6	4.5	89.7	8.0
Gill	20	92.6	5.2	92.4	7.1	96.5	2.9	95.6	1.9
	50	89.2	3.8	91.2	6.7	96.2	3.2	96.7	3.4
	100	93.8	3.6	84.6	3.3	94.8	2.9	89.0	6.2
Muscle	20	96.8	4.1	95.8	2.9	99.1	3.6	105.2	8.4
	50	94.1	5.3	95.4	4.2	86.4	1.1	83.9	1.8
	100	90.5	1.8	86.8	5.7	113.2	7.8	83.2	7.5
Gonad	20	91.4	5.9	93.2	9.1	98.2	7.9	84.1	6.1
	50	86.9	7.2	84.1	7.5	95.6	7.4	92.5	6.2
	100	87.6	1.4	84.6	5.2	92.9	6.3	93.8	4.8
Liver	20	96.5	9.2	94.9	6.8	94.8	4.2	84.6	9.7
	50	99.1	6.7	96.5	5.5	97.3	6.5	101.4	5.6
	100	101.3	11.1	93.6	3.8	97.1	9.8	95.6	11.9
Intestine	20	98.6	3.0	101.9	8.0	99.3	8.8	104.6	5.8
	50	89.7	6.6	97.1	9.8	95.8	10.1	95.6	9.0
	100	109.5	5.6	86.9	6.3	90.0	5.3	92.1	3.2

3.4. Tissue accumulation of bisphenol compounds in zebrafish

The concentration variation of bisphenol compounds in zebrafish tissues with and without MP was presented (Figure 3-3). At all treatment levels, the bisphenol compounds concentration in various tissues gradually increased with time and the summed concentrations varied from tissue to tissue. Moreover, within the 35d exposure

period, the accumulation of BPA and BPS had not yet reached a steady state. These results indicate the considerable capacity of zebrafish to accumulate bisphenol compounds[134].

Table 3-3. Analytical performance of the UPLC-MS/MS method for different tissues.

BPA					
Tissues	**Linearity range (μg/L)**	**Correlation coefficient (r)**	**Limit of detection (μg/L)**	**Limit of quantitation (μg/L)**	**Matrix effect (%)**
Brain	5.0-50.0	0.9984	0.3	1.0	5.6 ± 2.1
Gill	5.0-500.0	0.9994	0.3	1.0	8.8 ± 2.4
Muscle	5.0-500.0	0.9999	0.2	0.6	5.2 ± 1.6
Gonad	5.0-2000.0	0.9997	0.2	0.6	-9.4 ± 3.8
Liver	5.0-2500.0	0.9989	0.3	1.0	12.0 ± 2.7
Intestine	5.0-10000.0	0.9992	1.0	3.0	-11.2 ± 3.5
BPS					
Tissues	**Linearity range (μg/L)**	**Correlation coefficient (r)**	**Limit of detection (μg/L)**	**Limit of quantitation (μg/L)**	**Matrix effect (%)**
Brain	5.0-50.0	0.9990	0.3	1.0	13.9 ± 4.2
Gill	5.0-500.0	0.9992	0.3	1.0	5.7 ± 1.7
Muscle	5.0-500.0	0.9999	0.3	1.0	8.3 ± 1.1
Gonad	5.0-1000.0	0.9989	0.26	0.8	-4.8 ± 3.0
Liver	5.0-2000.0	0.9998	0.3	1.0	-15.6 ± 6.9
Intestine	5.0-10000.0	0.9993	1.0	3.0	-13.8 ± 5.2

The concentration of BPA and BPS in gill tissue were 12.38μg/L and 8.28μg/L at the first day and increased continuously during the accumulation period (Figure 3-3A). After 28d, the concentration levels of BPA and BPS showed an obvious increased, and at 35d, the concentration had reached 478.46μg/L and 105.10μg/L, respectively. In addition, the accumulation of BPA and BPS in gill were shown to be significantly boosted by the co-presence of MP, which were approximately at 596.11μg/L and 381.08μg/L after 35d. We also detected bisphenol compounds in brain (Figure 3-3B) and the concentration did not fluctuate at large level (BPA: 14.76μg/L; BPS: 15.47μg/L), but the presence of MP also lightly increased the accumulation of BPA and BPS (MA: 23.14μg/L; MS: 23.84μg/L).

In muscle (Figure 3-3C) and gonad (Figure 3-3D) tissues, the content of BPA were 360.10μg/L and 1646.27μg/L, respectively. The concentration of BPS in tissues were

319.94μg/L and 215.19μg/L at the end of exposure. Similarly, compared to single exposure, the co-exposure of MA and MS showed more accumulated in muscle and gonad tissues (Muscle: MA was 481.55μg/L and MS was 428.56μg/L; Gonad: MA was 1767.04μg/L and MS was 737.82μg/L). The maximum accumulation of BPA/BPS were detected in the intestine (Figure 3-3E) (8624.61μg/L and 6906.87μg/L, respectively), followed by the liver (Figure 3-3F) (2620.23μg/L and 1084.11μg/L, respectively). Also, there were 9654.02μg/L and 7457.46μg/L with MP in intestine, 2674.18μg/L and 1157.76μg/L with MP in liver after 35d exposure.

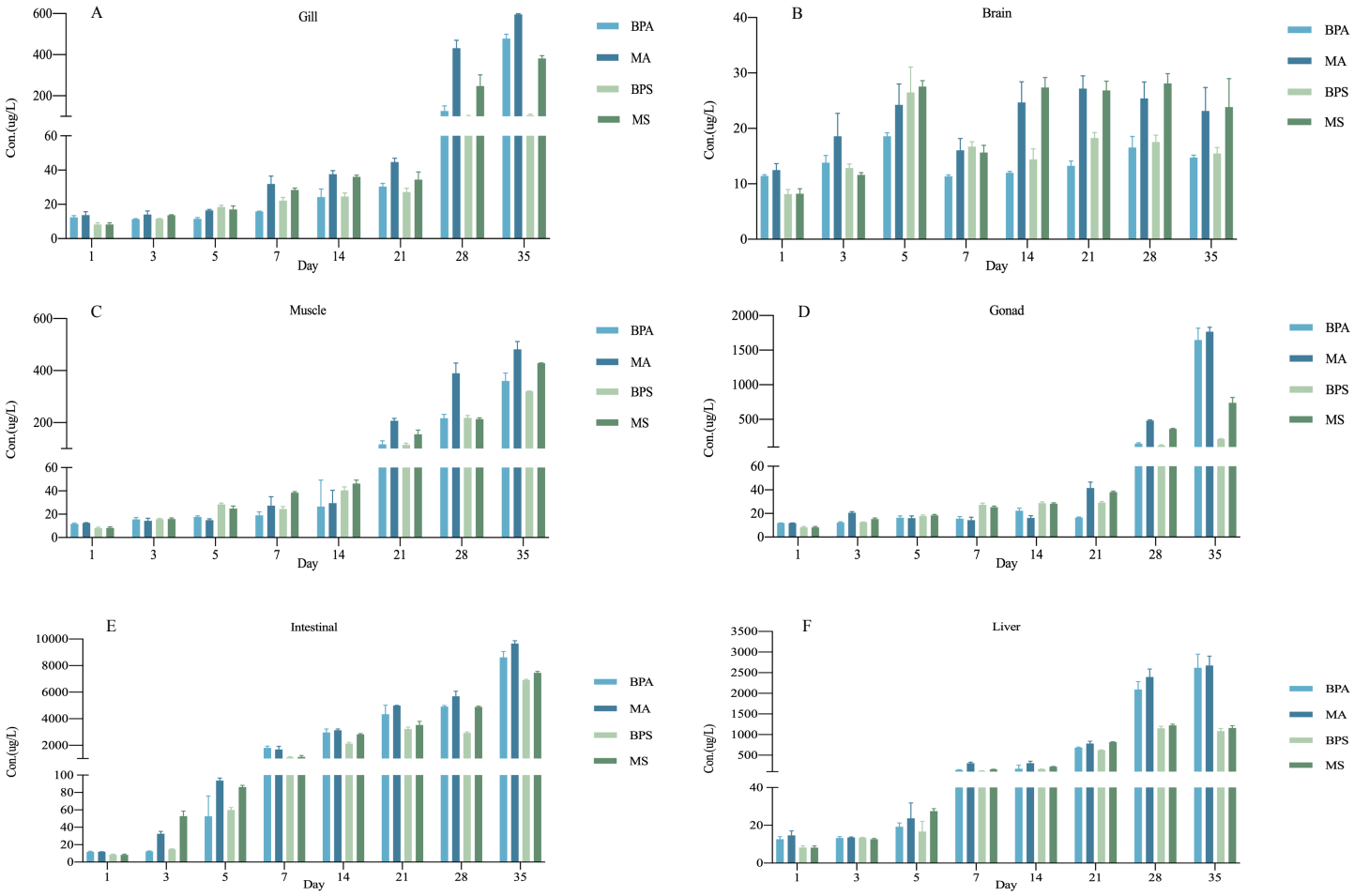

Figure 3-3. Tissue accumulation of bisphenol compounds with or without MP in different zebrafish tissues. (A): Gill; (B): Brain; (C): Muscle; (D): Gonad; (E): Intestine; (F): Liver. The y-axis represents the accumulation concentration of bisphenol compounds in zebrafish tissues, while the x-axis indicates the different exposure durations. Error bars indicate the standard deviation. All values are represented as mean ± standard derivation (n = 3 biological replicated).

4. Discussion

4.1 Optimization of sample pretreatment

Three kinds of SPE cartridges, namely EMR-Lipid, HLB and C_{18} were compared on their recoveries of bisphenol compounds in zebrafish tissues. EMR-Lipid, a unique absorbent that can specifically adopted for removal of lipids from biological samples[343,344]. EMR-Lipid was not suitable for extraction of BPA and BPS, with average recoveries lower than 60%, possible because of the low-fat content in zebrafish tissues. Next to it was HLB, for which the target analytes were eluted during the wash procedure. This may be because of some hydrophilic functional groups in HLB columns[345], of which were not suitable for extraction of BPA and BPS in zebrafish tissues. The HLB cartridges have lower extraction efficiency for bisphenol compounds has reported in elsewhere[346,347]. C_{18} SPE cartridges provided good extraction efficiencies for both analytes (72.9-109.5%), which could be owned to functional group of octadecyl in the C_{18} cartridges. Compared with HLB, the functional group of octadecyl in C_{18} cartridges possesses a high carbon content and offers strong hydrophobic interactions[345]. Therefore, C_{18} cartridges are more suitable for the extraction of BPA and BPS in zebrafish tissues and were used in subsequent experiments.

4.2 Tissue accumulation of bisphenol compounds in zebrafish

At present, the tissue-specific distribution of pollutants in zebrafish is frequently observed and utilized to further investigate the mechanism of toxicity[348]. In our study, BPA and its BPS analogue were detectable in zebrafish from first day. Rapid uptake and quick accumulation of bisphenol compounds have also been reported[308,349]. In addition, the concentrations of bisphenol compounds steadily increased over the 35-day exposure period, without reaching a steady state, highlights the potential for long-term bioaccumulation in aquatic organisms. And the higher tissue concentration of BPA and BPS were in viscera, such as intestine, liver and gonad, more than that in gill, muscle and brain. This distribution pattern likely results from xenobiotic transport processes and differences in lipid content among tissues, which play a crucial role in the bioaccumulation of hydrophobic compounds like bisphenols[136,308,350]. The liver, as the primary organ responsible for detoxification, is often a major site for the accumulation of xenobiotic compounds due to its role in metabolizing and excreting these substances[351]. The relatively high bisphenol concentrations found in this organ, as it acts as a central hub for chemical processing and storage, may explain the hepatotoxicity and disruption of lipid metabolism observed in response to bisphenol exposure[278,352,353].

As the initial absorption tissues, high accumulation levels of BPA and BPS were also found in intestine. That means zebrafish could be also exposed to bisphenol compounds in the environment directly through ingestion. The intestinal tissue, being in direct

contact with ingested bisphenols, acts as a primary site for accumulation before the compounds are distributed to other organs via the circulatory system[354]. Once bisphenols accumulated in intestine, they could be transferred to hemolymph and then distributed to other tissues along with the circulatory system[134], as observed in the present study.

In contrast, tissues with lower lipid content, such as gill, muscle and brain, exhibit comparatively lower accumulation of BPA and BPS. From direct contact with chemicals, fish could accumulate them from ambient water through respiration associating the gills to be priority organs to be exposed[355]. Bisphenol compounds are difficult to metabolize coming through gills, resulting in deposition[351]. Fish gills have shown significant accumulation of bisphenol compounds in the present study.

We speculated that the accumulation of bisphenol compounds in the brain further increased after co-exposure with MP because its lipophilic chemical structure leading to bisphenols cross over the blood-brain barrier[356] and reach the fish brain along with blood circulation. The existence of BPA and BPS in the zebrafish brain might be the reason why bisphenol compounds could injure the nervous system[271,357]. The residue of BPA and BPS in the gonad may disrupt ovarian redox balance and oocyte health[358]. Early research made it clear that BPA and its analogs could induced reproductive toxicity of zebrafish[33,359]. The variation in bisphenol accumulation across tissues also underscores the importance of understanding tissue-specific toxicokinetics when evaluating the potential health risks posed by these compounds.

The results of this study revealed that while both BPA and BPS accumulated in various tissues of zebrafish, the accumulation levels of BPS were consistently lower than those of BPA across all tissues. This difference in bioaccumulation may be a key factor underlying their differential toxic effects. Structurally, BPS is one of the most common analogs of BPA, with a similar chemical structure that allows it to function in a similar manner in biological systems[215]. Despite this structural resemblance, several studies have reported that BPS generally exerts similar or lower toxicities compared to BPA[325,357,359,360]. The lower accumulation of BPS observed in this study could provide insight into these differing toxicological profiles. For instance, Boucher et al. (2016) and Moreman et al. (2017) both suggested that the reduced bioavailability of BPS may result in weaker endocrine-disrupting effects compared to BPA, which aligns with our findings of lower BPS tissue concentrations[259,361]. The fact that BPS does not accumulate to the same extent as BPA in zebrafish tissues, especially in key organs such as the liver and intestine, where maximum concentrations were observed, suggests that it may be less prone to long-term retention and bioaccumulation, which in turn could reduce its chronic toxicity. The noticeably lower accumulation levels of BPS compared to BPA might provide a theoretical basis for understanding their differential toxic effects.

Finally in our study, the co-exposure with MP further facilitates BPA and BPS accumulation, especially in the gill, muscle, gonad, intestine and liver tissues. These results suggest that MP could enhance the bioavailability and uptake of bisphenol compounds, this posing a greater risk to aquatic life. For tissue accumulation, previous studies have reported that the presence of MP aggravate the bioaccumulation of environmental pollutants[322]. A series of recent studies revealed that the presence of MP may disrupt the detoxification process in organisms[325,362], potentially contributing to the aggravated accumulation of BPA and BPS in zebrafish. Another plausible explanation is that MP boost the accumulation of bisphenol compounds in different tissues of zebrafish through the Trojan horse effect[160,311,322]. The increased bioavailability and bioaccumulation of bisphenol compounds, facilitated by MP, might be the reason why MP could further aggravate their adverse impacts on neurotoxicity[308], immunotoxicity neurotoxicity[313] and reproductive toxicity[160] of organisms. Given the widespread environmental presence of both MP and bisphenol compounds, these findings have critical implications for the understanding of pollutant interactions and their combined effects on aquatic ecosystems.

Despite the significant findings, this study has limitations that must be acknowledged. First, while the accumulation patterns of bisphenol compounds were observed over 35 days, the lack of a steady state raises questions about the potential longer-term accumulation and effects beyond this period. Future studies should extend the exposure period to determine whether a steady-state concentration of bisphenol compounds can be reached and assess the chronic effects of prolonged exposure.

5. Conclusion

In this work the development of a sensitive and accuracy UPLC-MS/MS method for the simultaneously determination of BPA and BPS was described. This study is the first attempt to simultaneously determine two kinds of bisphenol compounds in different biological tissues. Good validation parameters were obtained in this work, laying a solid foundation for further analysis of the bioaccumulation and tissue distribution of bisphenol compounds in zebrafish. The developed method was successfully applied for the analysis of real zebrafish tissues samples. At the same time, the tissue distribution and accumulation of BPA and BPS alone or in combination with MP in zebrafish were investigated. We found that the accumulation concentration of BPS is lower than that of BPA, which might provide a theoretical basis for the understanding that BPS has lower toxicity compared to BPA. More importantly, the results revealed that the copresence of MP could aggravate the accumulation of BPA and BPS in different tissues of zebrafish. Thus, further investigation on the potential risks of co-exposure of MP and environmental pollutants to organisms and its underlying mechanisms of toxicity should be conducted.

Chapter 4

Toxicity effects of parental co-exposure of bisphenol compounds and microplastics on oxidative damage in zebrafish

Abstract

Researchers are increasingly studying the combined effects of microplastics and chemicals like BPA and BPS, commonly used as plasticizers, which are known to disrupt endocrine function and pose reproductive risks. Understanding these interactions is crucial due to potential impacts on ecosystems and organism health. However, significant gaps remain in understanding the exact toxic mechanisms and possible transgenerational effects of this combined exposure. Further investigation into these areas is essential for assessing and mitigating risks to both environment and human health. The results of this study indicate that adult zebrafish exposed to BPA and BPS, whether individually or in combination with microplastics, all resulted in significant damage to the liver and intestines, accompanied by pronounced oxidative stress and cell apoptosis. It is worth noting that offspring zebrafish not directly exposed to these compounds also exhibited significant developmental toxicity and oxidative damage, highlighting the transgenerational effects of BPA and BPS in zebrafish. In addition, melatonin treatment could partially restore oxidative damage in offspring zebrafish. In conclusion, this study contributes to a better understanding of the complex interactions between environmental pollutants and organisms, provides insights for species conservation efforts, and highlights the need for an integrated approach to assessing and managing environmental health risks.

Keywords: transgenerational toxicity, microplastics, bisphenol A, bisphenol S, zebrafish

1. Introduction

In recent years, concerns over the environmental and health impacts of plastic-derived chemicals have garnered increasing attention worldwide. Among these chemicals, bisphenol A (BPA) and bisphenol S (BPS) have been extensively studied due to their widespread use in consumer products and potential adverse effects on human health and aquatic ecosystems[363,364]. BPA and BPS are commonly found in plastics, epoxy resins, and thermal paper, leading to widespread environmental contamination. Furthermore, the presence of microplastics (MP), defined as plastic particles smaller than 5mm, has emerged as a significant environmental issue. MP are generated from the fragmentation of larger plastic debris or are intentionally manufactured for various industrial and consumer applications[365,366].

At a hazardous waste site, the concentration of BPA reached a significant level of 17.2mg/L[367]. Additionally, BPS has been found in river water with concentrations reaching up to 3μg/L[368]. In inland surface waters, there is a significant coexistence of MP and bisphenol analogues, as they are both commonly found pollutants. For instance, in the Guangzhou section of the Pearl River and the Pearl River Estuary, the detection content of MP reached 19,860/m^3 and 8902/m^3 respectively[33]. Similarly, the concentration of MP in samples from Taihu Lake, the third largest freshwater lake in China, ranged from 3400 to 25,800/m^3. Furthermore, these areas also exhibit high detection frequencies of bisphenol pollutants, with BPA and BPS being detected in Taihu Lake and the Pearl River[369].

The simultaneous exposure to BPA, BPS and MP has raised concerns regarding their combined effects on aquatic organisms. Among these organisms, zebrafish have been widely used as a model organism in ecotoxicology due to their sensitivity to environmental stressors and genetic similarity to humans[370–372]. Previous studies have demonstrated that exposure to BPA and BPS individually can induce oxidative stress, disrupt endocrine function, and lead to developmental abnormalities in zebrafish[372–374]. Similarly, MP exposure has been associated with adverse effects on zebrafish health, including impaired growth, altered behavior, and tissue damage. However, limited research has investigated the combined effects of BPA, BPS, and MP on oxidative damage in zebrafish[375].

Oxidative stress, characterized by an imbalance between the production of reactive oxygen species (ROS) and antioxidant defense mechanisms, is a key mechanism underlying the toxicity of environmental contaminants. ROS, including superoxide radicals, hydrogen peroxide, and hydroxyl radicals, can cause damage to lipids, proteins, and nucleic acids, leading to cellular dysfunction and tissue injury[370,376,377]. Given the potential for BPA, BPS, and MP to induce oxidative stress individually, there is a critical need to elucidate their combined effects on zebrafish oxidative damage.

The objective of this study was to investigate the effects of BPA and BPS exposure, both individually and in combination with MP, on the health of adult zebrafish and their offspring. Specifically, we aimed to assess the potential damage to the liver and intestines, evaluate oxidative stress levels, examine cellular apoptosis, and explore transgenerational effects. Furthermore, we aimed to explore potential mitigation strategies, such as melatonin treatment, to alleviate any observed adverse effects. Overall, the goal was to provide comprehensive insights into the toxicity of BPA and BPS in zebrafish and their interactions with MP, contributing to a better understanding of environmental health risks.

2. Materials and Methods

2.1 Chemicals

99% bisphenol A (CAS: 80-05-7) and 99% bisphenol S (CAS: 80-09-1) were purchased from Aladdin Biochemical Technology Co., Ltd (Shanghai, China). Polyethylene microplastics with particle size of 2μm were purchased from Zhichuan Technology Co., LTD (Jiangsu, China). All other chemicals and reagents utilized in this study were of analytical grade and used without further purification.

2.2 Experimental fish

Zebrafish (Danio rerio, AB-wild type, aged 5 months) were purchased from the aquarium department of Hongdagaofeng. Healthy adult fish aged 5 months were individually selected and acclimated in glass tanks (4 L) under a constant temperature of 25.0 ± 1.0 °C for two weeks with a photoperiod of 14 hours light and 10 hours dark. The fish were fed with Fairy Shrimp two times daily during the acclimatization period.

2.3 Fish exposure and sample collection

The exposure of adult zebrafish was divided into six groups: the control group without drug exposure, the group exposed to 100μg/L microplastics, the group exposed to 100μg/L BPA, the group exposed to 100μg/L BPS, the group exposed to 100μg/L microplastics with 100μg/L BPA, and the group exposed to 100μg/L microplastics with 100μg/L BPS. The drug exposure duration was 35 days. After exposure, 5 fish were randomly selected from each treatment group for histopathological analysis, 6 fish were used as one sample for the determination of oxidative damage-related indicators, and 8 fish were allowed to spawn for subsequent experiments. Each experiment was conducted in triplicate.

After spawning, zebrafish embryos were not subjected to any drug exposure. The number of embryos used for morphological observation was 20, for analysis of oxidative damage-related indicators was 30, and for melatonin repair and subsequent

determination was 30. Each experiment was conducted in triplicate. The experiment time was up to 96hpf. The solutions were changed once every 24h at which time any dead embryos were discarded. After 96h of experiment, the zebrafish embryos were collected, immediately frozen in liquid nitrogen, and finally stored at −80 °C for further analysis.

2.4 Histopathological analysis

The excised liver and intestine specimens were initially fixed in 4% formalin and subsequently underwent sequential processing for paraffin embedding, involving dehydration with ethanol, clearing with xylene, and embedding in paraffin. The tissue samples mounted on slides were then sectioned into slices measuring 4μm in thickness. These sections were stained with eosin and hematoxylin, followed by air-drying. Images were captured using an Olympus DP71 camera and software under a magnification of 400X.

2.5 Analysis of antioxidant levels in adult zebrafish

Liver samples and intestine samples, after thawing, were homogenized on ice using a tissue homogenizer (Tiangen, Beijing, China) in 10 volumes of cold phosphate buffer (5 mM, pH 7.8). Following centrifugation at 2000 × g at 4 °C for 10 min, the resulting supernatant was collected for enzyme activity assays. The activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), as well as the glutathione (GSH) content, were determined using test kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). All presented results represent data from three independent experiments.

2.6 RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

Zebrafish Liver samples and intestine samples were collected and total ribonucleic acid (RNA) was extracted using TRIzol reagent (Tiangen Biotech, Beijing, China). Total RNA quality and concentrations were assessed with a DS-11 spectrophotometer (DeNovix, USA), and 1.5μg of RNA was employed for cDNA synthesis in a 20μL reaction utilizing the FastQuant RT Kit (Tiangen, Beijing, China). The qRT-PCR was carried out in a 20μL reaction volume using an ABI 7500 system (Advanced Biosystems, Foster City, CA, USA) with conditions set at 95°C for 15 min, followed by 40 cycles of amplification at 95°C for 10s, 60°C for 20s, and a melting curve analysis at 72°C for 32s.

Gene expression differences between exposure groups and control groups were evaluated using the $2^{-\Delta\Delta CT}$ method, with *β-actin* as the reference gene and appropriate

negative controls included for all primers. Zebrafish primer sequences can be found in the Table 4-1.

2.7 Zebrafish embryo experiment

For each treatment group, both untreated and 100μg/L melatonin-exposed groups were established, with the experimental duration set at 96 hours post-fertilization (hpf). Observations were conducted using a standard stereomicroscope (Egtech, Beijing, China) to assess spontaneous movements within a 20-second interval at 24hpf and heartbeats within the same timeframe at 48hpf. The hatching rate was calculated at 72hpf. Post-exposure, embryos underwent the determination of oxidative damage-related indicators, employing the same experimental protocols detailed in Section 2.5.

Table 4-1. Primer sequence for the quantitative reverse transcription-polymerase chain reaction used in this study

Gene	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')
p53	GGGCAATCAGCGAGCAAA	ACTGACCTTCCTGAGTCTCCA
bax	GGCTATTTCAACCAGGGTTCC	TGCGAATCACCAATGCTGT
apaf-1	TTCTACAGTAAACGCCCACC	TATCTAGTATTTCCCCATATTCC
caspaes-3	CCGCTGCCCATCACTA	ATCCTTTCACGACCATCT
caspaes-9	AAATACATAGCAAGGCAACC	CACAGGGAATCAAGAAAGG
bcl-2	TCACTCGTTCAGACCCTCAT	ACGCTTTCCACGCACAT
puma	TGGAAAGCAGAGTGGACGAA	GATGGCAGGGCTGGATGA

2.8 Statistical analysis

Statistical analysis was conducted by using SPSS 17.0 software. Data were shown as the mean ± standard error of the mean (SEM). The differences between groups were analyzed by one-way analysis of variance (ANOVA) followed by a post hoc least significant difference (LSD) test. $P < 0.05$ was statistically significant difference.

3. Results

3.1 Histopathological analysis

Histopathological changes in zebrafish liver and intestine were observed using H&E staining, as shown in Figure 4-1. Compared to the control group, all drug-exposed

groups showed significant liver damage, including vacuolization and hepatocyte swelling, as well as increased inflammatory cells. Compared to the group exposed to MP alone, the BPA, BPS, and combined exposure groups exhibited more pronounced liver damage. The difference in liver damage between the BPA, BPS alone, and combined exposure groups with MP in zebrafish was not significant.

For the intestine, the situation is different. No significant changes were observed in the BPA and BPS single-exposure groups compared to the control group. However, the group exposed to MP showed significant phenomena such as distortion and shortening of intestinal villi, destruction of crypt structures, and a decrease in their number. Intestinal damage was more severe after combined exposure with BPA and BPS.

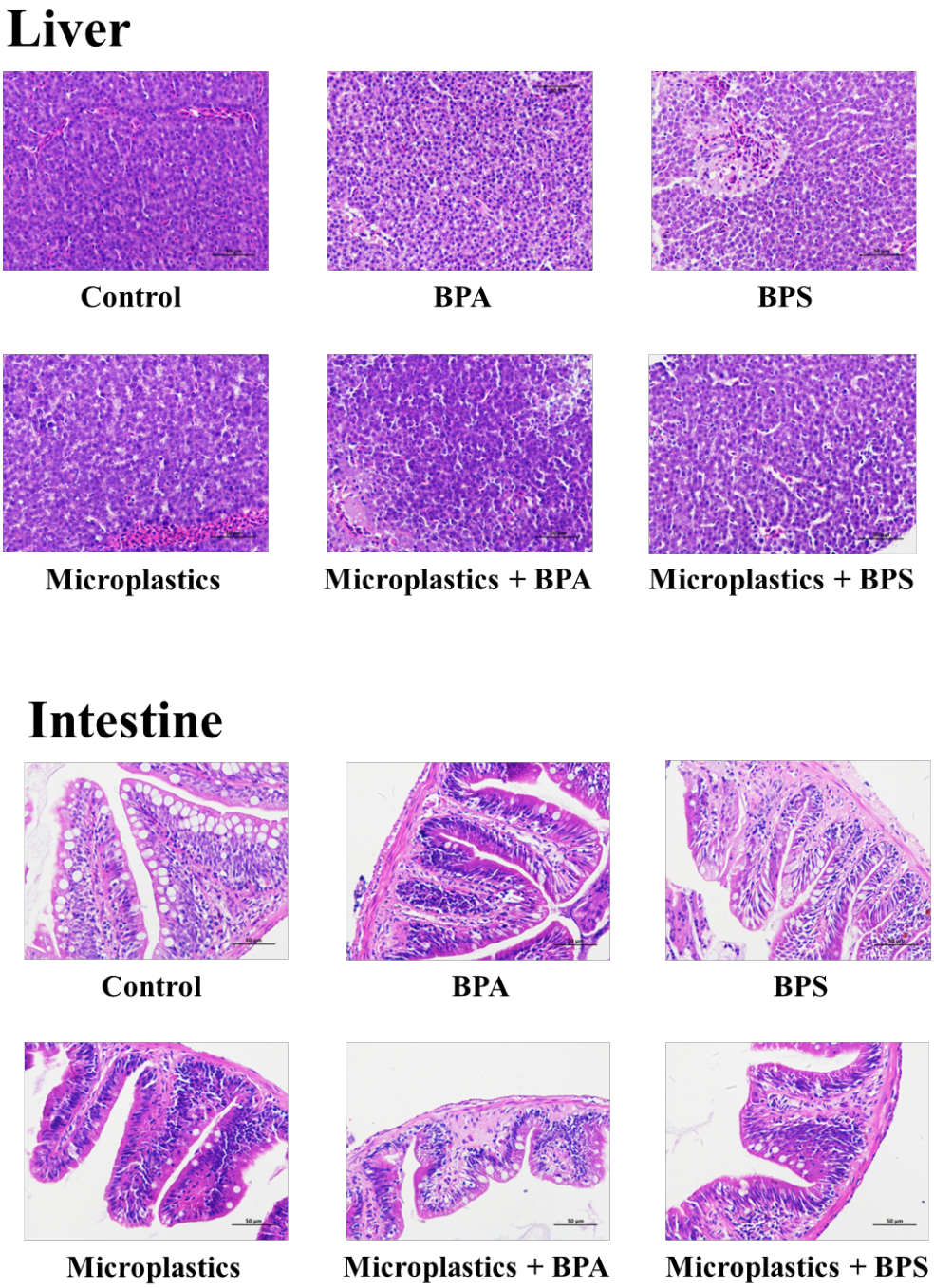

Figure 4-1. Histological changes of liver and intestine in zebrafish.

3.2 Results of oxidative damage detection

After drug exposure, oxidative stress-related indicators, including the activities of SOD, CAT, and GPx, as well as the content of GSH, were measured in the liver and intestine of zebrafish, as shown in Figure 4-2 and Figure 4-3.

Regarding the liver, the results revealed that compared to the control group, both the BPA and BPS single-exposure groups and the combined exposure group with microplastics exhibited significant downregulation of SOD activity, while the group

exposed to MP alone showed no change in SOD activity. Furthermore, compared to the single-exposure groups, a decreasing trend in SOD activity was observed after combined exposure to BPA, BPS, and MP, although no significant changes were noted. For CAT and GPx activities, the results were similar to SOD, wherein compared to the control group, both the BPA and BPS single-exposure groups and the combined exposure group with MP exhibited significant downregulation, while the group exposed to MP alone showed no change. The content of GSH did not show significant changes in any of the treatment groups.

For the intestine, the results were similar to the liver. Compared to the control group, both the BPA and BPS single-exposure groups and the combined exposure group with MP exhibited significant downregulation of SOD, CAT, and GPx activities, while the group exposed to MP alone showed no significant changes. The content of GSH did not show significant changes in any of the treatment groups.

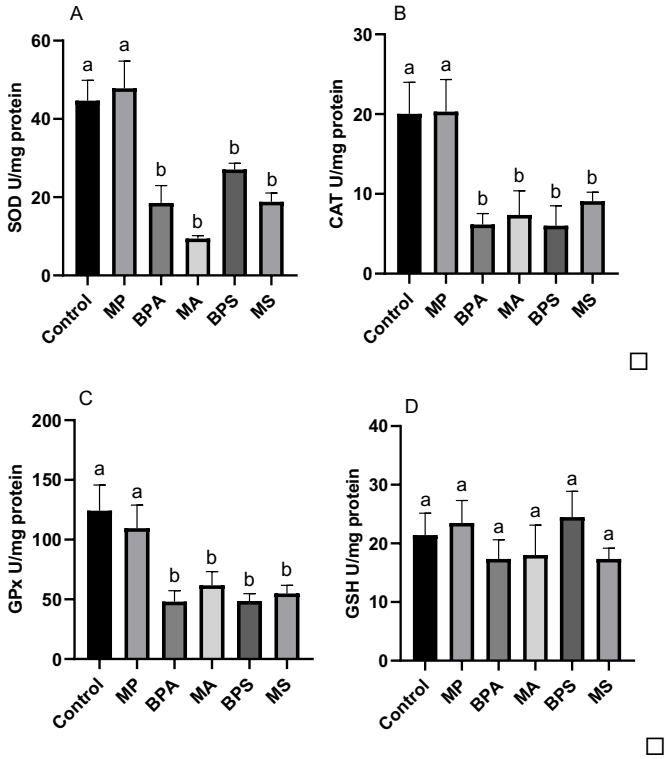

Figure 4-2. SOD (A), CAT (B), GPx (C) activities as well as GSH content (D) in liver of adult zebrafish. The data were analyzed by ANOVA test. ($p<0.05$)

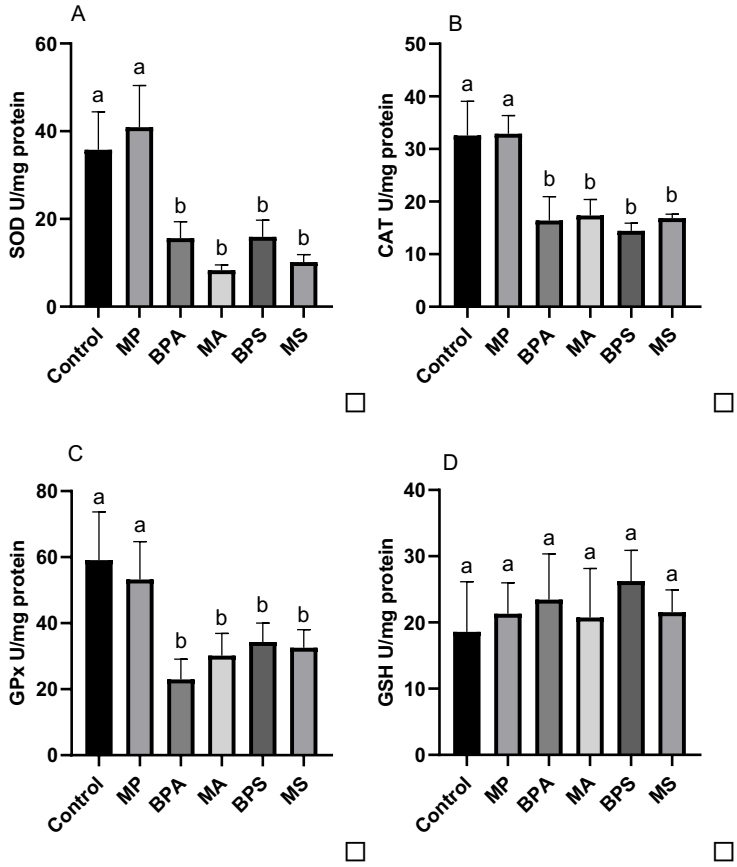

Figure 4-3. SOD (A), CAT (B), GPx (C) activities as well as GSH content (D) in intestine of adult zebrafish. The data were analyzed by ANOVA test. ($p<0.05$)

3.3 Gene expression related to apoptosis

To investigate the impact of BPA, BPS, and MP exposure on apoptosis in zebrafish liver and intestinal cells, we assessed the expression levels of several genes including *p53*, *puma*, *apaf-1*, *bax*, *caspase-3*, *caspase-9* and *bcl-2*, as shown in Figure 4-4 and 4-5.

In the liver, compared to the control group, all gene expressions remained unchanged in the group exposed to MP alone. Regarding *p53* gene expression, significant upregulation was observed in both the BPA and BPS single-exposure groups as well as the combined exposure group with MP, compared to the control group. Specifically, significant differences in expression levels were observed between the BPA single-exposure group and the combined exposure group with MP. For the *puma* gene, significant upregulation was observed in the BPA combined with MP exposure group, the BPS single-exposure group, and the combined exposure group with MP, while exposure to BPA alone did not alter its expression levels. Similar results were observed for *apaf-1*, *bax*, *bcl-2*, and *caspase-9*, where significant upregulation in expression levels was observed in both the BPA and BPS single-exposure groups as well as the combined exposure group with MP, compared to the control group. No significant changes in *caspase-3* gene expression were observed due to drug exposure.

The intestinal condition is similar to but slightly different from that of the liver. In the intestine, no significant changes were observed in gene expression in the group exposed solely to MP. In comparison to the control group, a significant upregulation in the expression levels of the *p53* gene was observed in the groups exposed to a combination of BPA and MP, as well as in the groups exposed solely to BPS and in combination with MP while the group exposed solely to BPA showed no significant changes. No significant changes in the expression of the *puma* gene were observed due to drug exposure. Similar results were observed for *apaf-1*, *bax*, *bcl-2*, and *caspase-9* genes, where a significant upregulation in expression levels was observed in the groups exposed solely to BPA or BPS and in combination with MP compared to the control group. In the intestine, the expression of the *caspase-3* gene also changed following drug exposure, with a significant upregulation observed in the groups exposed solely to BPA, in combination with MP, and in combination with BPS and MP compared to the control group.

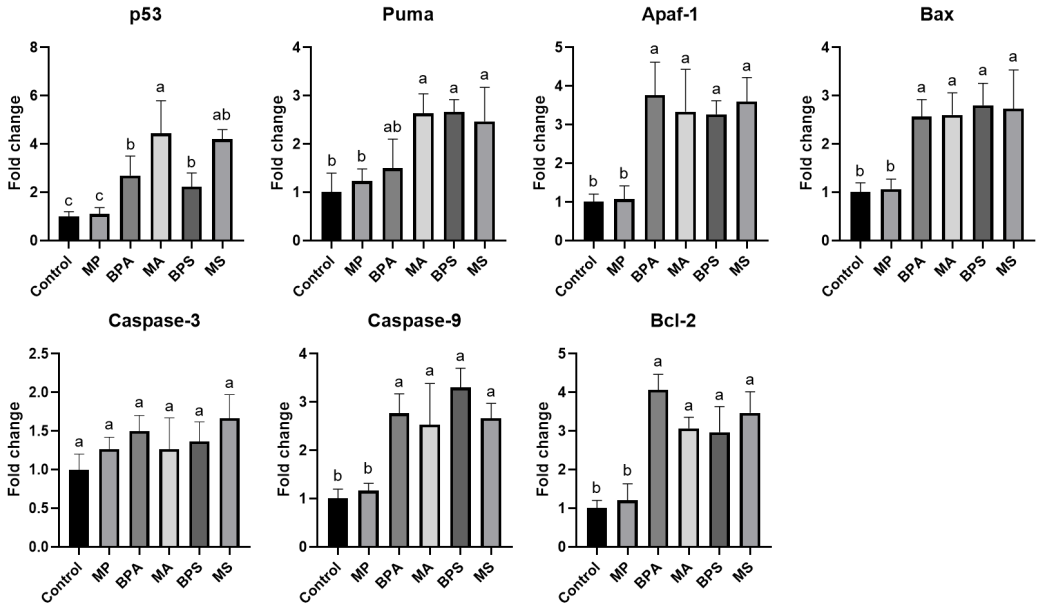

Figure 4-4. Expression of genes in liver of adult zebrafish. The data were analyzed by ANOVA test. ($p<0.05$)

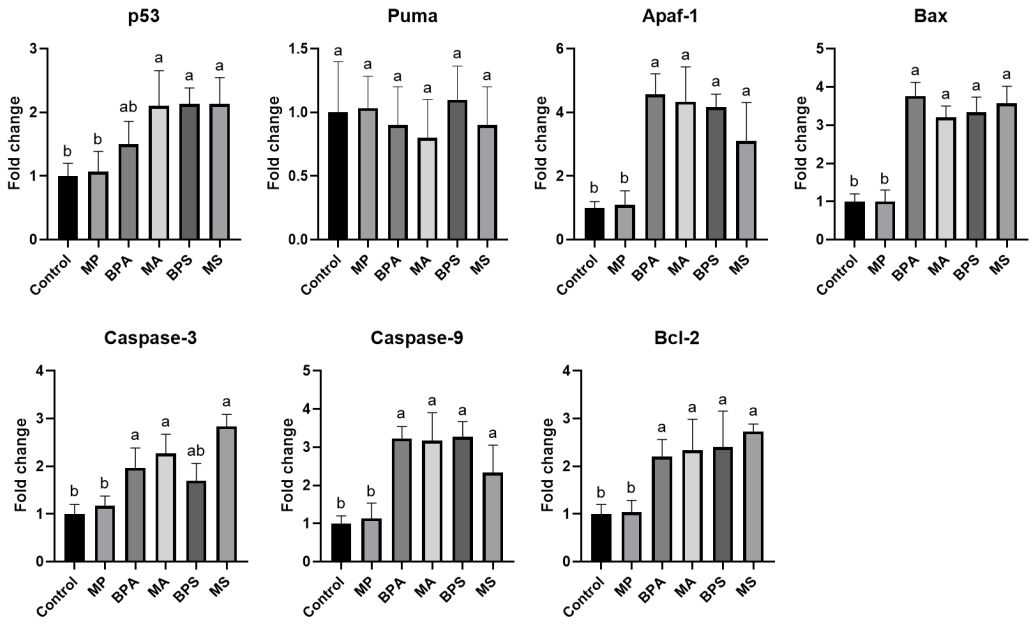

Figure 4-5. Expression of genes in intestine of adult zebrafish. The data were analyzed by ANOVA test. ($p<0.05$)

3.4 Developmental toxicity and oxidative damage in zebrafish embryos

In order to evaluate the potential toxicity of drug exposure in adult zebrafish on the development of their offspring, we assessed zebrafish larvae for spontaneous movement, heart rate, hatching rate, and malformation rate, as depicted in Figure 4-6. Concurrently, given the evident oxidative damage caused by drug exposure in adult zebrafish, which often accompanies developmental toxicity in zebrafish larvae, we also measured oxidative stress-related parameters, including the activities of SOD, CAT, and GPx, as well as the content of GSH, as illustrated in Figure 4-7.

Observations of zebrafish embryos at 24hpf revealed that exposure to MP alone did not significantly affect the spontaneous movement of their offspring compared to the control group. However, both BPA and BPS exposure alone, as well as combined exposure with MP in adult zebrafish, led to a significant increase in the frequency of spontaneous movement in their offspring. At 48hpf, zebrafish embryo heart rates were measured, showing that exposure to MP alone did not significantly affect the heart rates of their offspring. However, offspring of adult zebrafish exposed to BPA alone, BPS alone, or in combination with MP exhibited a significant decrease in heart rate. The group exposed to a combination of BPA and MP showed a decrease in heart rate, although not statistically significant. The hatching rate was assessed at 72hpf, revealing that drug exposure in parental zebrafish did not affect the hatching of their offspring, as embryos from all treatment groups hatched almost entirely without significant differences. Finally, we analyzed the malformations of zebrafish embryos at 72hpf and found that certain treatment groups exhibited typical deformities such as yolk sac

edema and spinal curvature. Statistical analysis of malformation rates indicated that only combined exposure to BPS and MP significantly increased the malformation rate in zebrafish larvae offspring. Malformation rates in the remaining treatment groups showed an increase, albeit not statistically significant.

The oxidative stress-related indicators in the offspring zebrafish larvae exhibited similarities to those of adult zebrafish. Relative to the control group, significant reductions in SOD activity were observed in the offspring zebrafish exposed to BPA, BPS individually, and in combination with MP. Conversely, the group exposed solely to MP showed no alteration in SOD activity. Similar trends were observed for CAT and GPx activity, mirroring those of SOD. Specifically, significant decreases were noted in the groups exposed to BPA, BPS individually, and in combination with MP compared to the control group, while no changes were observed in the group exposed solely to microplastics. The GSH content did not exhibit significant changes across all treatment groups.

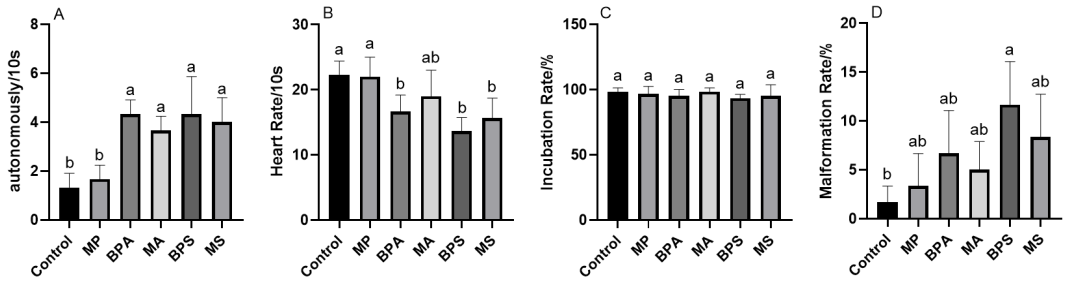

Figure 4-6. Effects of BPA, BPS and microplastics on embryonic development. A. Autonomic movement at 24hpf. B. Heart rate at 48hpf. C. Hatching rate at 72hpf. D. Malformation rate at 72hpf. The data were analyzed by ANOVA test. ($p<0.05$)

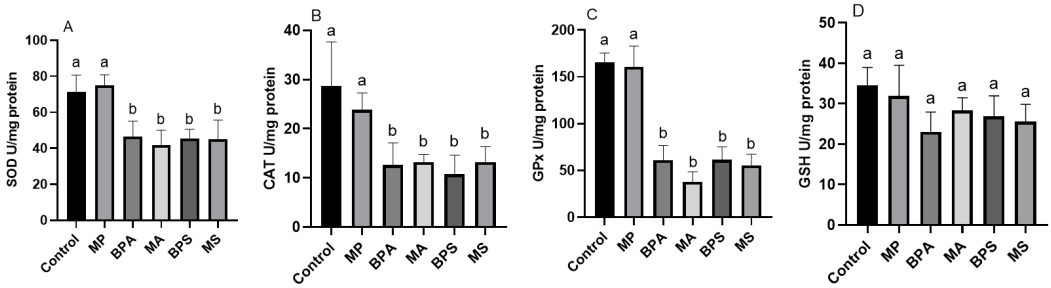

Figure 4-7. SOD (A), CAT (B), GPx (C) activities as well as GSH content (D) in zebrafish embryos. The data were analyzed by ANOVA test. ($p<0.05$)

3.5 Melatonin repair study

In order to investigate the potential mitigating effects of melatonin on the developmental toxicity and oxidative damage induced by BPA and BPS in zebrafish

embryos, we conducted exposure experiments with melatonin on zebrafish embryos. The results are presented in Figure 4-8.

Observations of spontaneous movement of zebrafish embryos at 24hpf indicated a decreasing trend in locomotor activity when co-exposed to melatonin compared to individual exposures to BPA or BPS, although statistical significance was not achieved. Similar trends were observed in heart rate at 48hpf and malformation rates at 72hpf, with melatonin exhibiting some degree of ameliorative effects, albeit not statistically significant.

However, measurements of oxidative stress-related markers revealed a clear reparative effect of melatonin on oxidative damage in zebrafish embryos. Compared to the control group, exposure to BPA and BPS individually resulted in a significant decrease in SOD activity in offspring zebrafish larvae, which was restored to levels comparable to the control group upon co-exposure to melatonin. Moreover, the addition of melatonin led to a significant increase in SOD activity compared to the non-melatonin-treated BPA exposure group. Changes in CAT and GPx enzyme activities were even more pronounced; offspring zebrafish larvae exposed to BPA or BPS individually exhibited a significant decrease in CAT and GPx enzyme activities compared to the control group, which were restored to levels comparable to the control group upon co-exposure to melatonin, and differed significantly from the non-melatonin-treated exposure group.

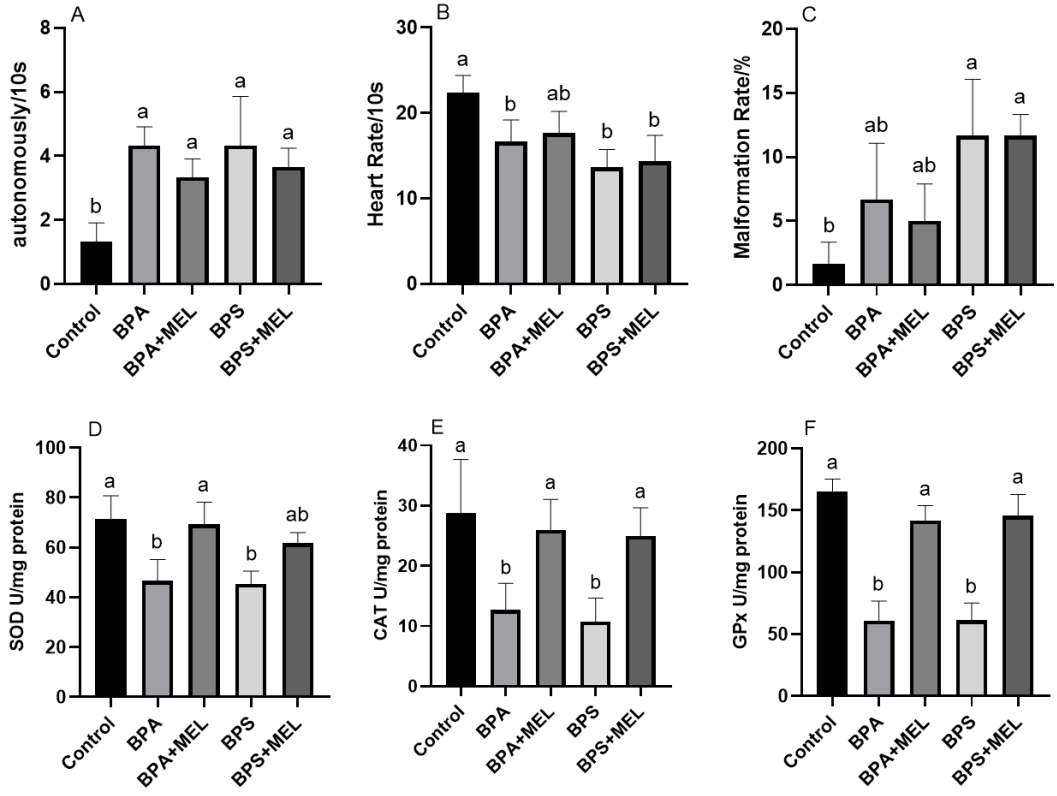

Figure 4-8. Indicators of embryonic development and oxidative damage in zebrafish after melatonin exposure. The data were analyzed by ANOVA test. ($p<0.05$)

4. Discussion

In this study, we evaluated the oxidative damage to the liver and intestine of adult zebrafish caused by combined exposure to BPA, BPS, and MP. We investigated the developmental toxicity and oxidative damage in adult zebrafish and offspring. Finally, melatonin was employed to repair the oxidative damage in zebrafish embryos.

Based on the histopathological analysis conducted in this study, significant liver damage was observed in all drug-exposed groups compared to the control group, characterized by vacuolization, hepatocyte swelling, and increased inflammatory cells. Furthermore, the severity of liver damage was notably exacerbated in the BPA, BPS, and combined exposure groups compared to the MP alone group. However, the differences in liver damage among the BPA, BPS alone, and combined exposure groups with MP did not reach statistical significance. Contrastingly, the intestinal histopathological changes exhibited distinct patterns. While no significant alterations were noted in the BPA and BPS single-exposure groups compared to the control group, significant abnormalities were observed in the group exposed to MP, including distortion and shortening of intestinal villi, destruction of crypt structures, and a reduction in their number. Remarkably, the intestinal damage became more severe following combined exposure to BPA and BPS. These findings underscore the differential susceptibility of zebrafish liver and intestine to various chemical exposures, suggesting that the combined exposure to BPA and BPS may exacerbate intestinal damage while exerting a more pronounced effect on liver histopathology[378,379]. The observed histopathological alterations provide valuable insights into the potential mechanisms underlying the developmental toxicity induced by these chemicals and highlight the importance of assessing multiple organ systems in toxicity studies.

The alterations in oxidative stress-related indicators in both the liver and intestine of zebrafish following drug exposure prompt several significant discussions. Firstly, the observed downregulation of SOD, CAT, and GPx activities in the liver and intestine of zebrafish indicates a disturbance in the antioxidant defense system upon exposure to BPA, BPS, and MP, either individually or in combination. This disruption suggests an imbalance between the production of reactive oxygen species (ROS) and the antioxidant capacity of the organism, leading to oxidative stress. The downregulation of these antioxidant enzymes, which play crucial roles in neutralizing ROS, highlights the susceptibility of zebrafish to environmental contaminants and underscores the potential adverse effects on their health. Moreover, the differential response in SOD activity between the single-exposure and combined exposure groups suggests a possible interactive effect among BPA, BPS, and MP. While the individual exposures led to significant downregulation of SOD activity, the combined exposure showed a decreasing trend without reaching statistical significance. This trend hints at a potential synergistic or additive interaction among these contaminants, wherein their combined

presence may exacerbate the oxidative stress response compared to individual exposures alone. Further investigation into the mechanistic basis of these interactions is warranted to elucidate the underlying pathways and potential cumulative effects on zebrafish health[263,380,381]. Furthermore, the lack of significant changes in GSH content across all treatment groups warrants attention. Glutathione is a vital intracellular antioxidant that participates in detoxification processes and helps maintain redox homeostasis. The stable levels of GSH despite alterations in enzyme activities suggest a compensatory mechanism to mitigate oxidative damage. However, the exact mechanisms underlying this compensatory response and its implications for overall oxidative stress resilience remain unclear and warrant further investigation[382,383]. In summary, the findings underscore the susceptibility of zebrafish to oxidative stress induced by BPA, BPS, and MP, with potential interactive effects among these contaminants. Understanding the complex interplay between environmental stressors and antioxidant defense mechanisms is crucial for assessing the health risks associated with chemical exposures and developing targeted interventions to mitigate their adverse effects on aquatic organisms and ecosystems.

The results indicate differential gene expression patterns in response to the various exposures. While MP exposure alone did not elicit significant changes in gene expression compared to the control group, both BPA and BPS single exposures, as well as their combined exposure with MP, led to significant alterations in several apoptosis-related genes. Specifically, upregulation of *p53*, *puma*, *apaf-1*, *bax*, *bcl-2*, and *caspase-9* genes suggests activation of apoptotic pathways in response to these chemical exposures. The observed upregulation of apoptosis-related genes suggests a potential activation of apoptotic pathways in zebrafish liver and intestinal cells following exposure to BPA, BPS, and MP. Apoptosis, or programmed cell death, plays a crucial role in maintaining tissue homeostasis and eliminating damaged or abnormal cells. However, dysregulation of apoptosis can lead to pathological conditions and adverse health effects. Therefore, the observed alterations in gene expression patterns raise concerns regarding the potential toxicological effects of these environmental contaminants on zebrafish health[368,384,385]. The observed significant differences in gene expression between the BPA single-exposure group and the combined exposure group with MP further highlight the potential modulatory effects of MP on BPA-induced apoptosis pathways. Interestingly, while exposure to BPA alone did not affect *puma* gene expression, significant upregulation was observed in the BPA combined with MP exposure group, indicating a potential synergistic effect between BPA and MP on *puma* gene regulation. This suggests that the presence of MP may exacerbate the apoptotic response induced by BPA exposure. Moreover, the consistent upregulation of apoptosis-related genes across BPA, BPS, and combined exposure groups underscores the potential harmful effects of these environmental pollutants on zebrafish liver cells. The absence of significant changes in *caspase-3* gene expression suggests a complex regulation of apoptotic pathways, wherein specific components may be more sensitive

to chemical exposures than others. In conclusion, our study provides evidence of the impact of BPA, BPS, and MP exposure on apoptosis regulation in zebrafish liver and intestinal cells. These findings contribute to our understanding of the mechanisms underlying the toxicity of environmental contaminants and emphasize the need for further research to elucidate the molecular pathways involved. Ultimately, such knowledge is essential for developing effective strategies to mitigate the adverse effects of chemical pollutants on aquatic ecosystems and human health.

The study investigated the potential developmental toxicity induced by drug exposure in adult zebrafish and its impact on the offspring. We assessed various developmental endpoints in zebrafish larvae, including spontaneous movement, heart rate, hatching rate, and malformation rate, as well as oxidative stress-related parameters. Spontaneous movements of zebrafish embryos serve as an indicator for assessing the developmental toxicity and neurotoxic potential of chemical compounds[386]. The observed increase in spontaneous movement frequency in offspring from adult zebrafish exposed to BPA and BPS, either alone or in combination with MP, suggests they had neurotoxicity to the offspring. This finding aligns with previous studies linking developmental exposure to BPA and BPS with behavioral alterations in zebrafish larvae[387]. However, the absence of a significant effect on spontaneous movement in offspring from adult zebrafish solely exposed to MP indicates a differential impact of these pollutants on larval behavior. Furthermore, the significant decrease in heart rate observed in offspring from adult zebrafish exposed to BPA and BPS, either alone or in combination with MP, raises concerns regarding cardiac development. Cardiac function is crucial for embryo survival and normal development, and alterations in heart rate may indicate cardiac developmental defects induced by drug exposure in parental zebrafish. While the decrease in heart rate was not statistically significant in the group exposed to a combination of BPA and MP, the trend suggests a potential additive effect of these pollutants on cardiac function in offspring[385,388]. The consistent hatching rate across all treatment groups indicates that drug exposure in parental zebrafish did not significantly impair embryo viability or hatching success. However, the presence of typical deformities such as yolk sac edema and spinal curvature in certain treatment groups underscores the potential teratogenic effects of drug exposure on embryonic development. Specifically, the significant increase in malformation rate observed in offspring from adult zebrafish exposed to BPS in combination with MP highlights the interactive effects of these pollutants on developmental outcomes. The parallel changes in oxidative stress-related parameters between adult zebrafish and their offspring suggest a transgenerational impact of drug exposure on redox homeostasis. The reduction in SOD, CAT, and GPx activities in offspring exposed to BPA and BPS, either alone or in combination with MP, reflects an imbalance in antioxidant defense mechanisms, predisposing embryos to oxidative damage. Interestingly, the absence of alterations in SOD activity in offspring solely exposed to MP suggests differential susceptibility to oxidative stress among pollutant types. In conclusion, our findings

demonstrate the developmental toxicity of drug exposure in adult zebrafish on their offspring, as evidenced by alterations in developmental endpoints and oxidative stress-related parameters. These results underscore the importance of understanding the potential transgenerational effects of environmental pollutants on offspring health and highlight the need for further research to elucidate underlying mechanisms and inform regulatory policies aimed at minimizing developmental risks associated with chemical exposures.

The findings from our study provide valuable insights into the potential mitigating effects of melatonin on the developmental toxicity and oxidative damage induced by BPA and BPS in zebrafish embryos. Melatonin, an endogenous hormone synthesized by the pineal gland, plays a critical role in regulating various physiological functions, including sleep-wake cycles, circadian rhythms, and neuroendocrine activity[389]. Beyond its physiological roles, melatonin exhibits significant pharmacological properties, particularly as a potent antioxidant. It possesses anti-inflammatory and anti-apoptotic capabilities, contributing to cellular protection[390]. Melatonin and its metabolites effectively scavenge reactive oxygen species (ROS) and reactive nitrogen species (RNS), thereby preventing oxidative damage to mitochondria[391,392]. Furthermore, melatonin enhances the activity of several antioxidant enzymes, playing a crucial role in maintaining cellular redox homeostasis and mitigating the effects of aging[393,394]. While in our study, the behavioral and developmental observations did not reach statistical significance, indicating a decreasing trend in locomotor activity, heart rate, and malformation rates in zebrafish embryos co-exposed to melatonin with BPA or BPS compared to individual exposures, the trend suggests a potential ameliorative effect of melatonin on these endpoints. Further investigation with larger sample sizes may be warranted to confirm these trends and establish statistical significance. Of particular significance are the observations regarding oxidative stress-related markers. Our results clearly demonstrate that melatonin has a reparative effect on oxidative damage induced by BPA and BPS in zebrafish embryos. Specifically, co-exposure to melatonin with BPA or BPS restored SOD activity to levels comparable to the control group, indicating a mitigation of oxidative stress. Moreover, the addition of melatonin resulted in a significant increase in SOD activity compared to the non-melatonin-treated BPA exposure group, suggesting a potential enhancement of antioxidant defense mechanisms. Furthermore, the changes observed in CAT and GPx enzyme activities underscore the significant protective effects of melatonin against oxidative damage. Co-exposure to melatonin with BPA or BPS restored CAT and GPx enzyme activities to levels comparable to the control group, indicating an enhancement of antioxidant enzyme function in mitigating oxidative stress. These findings highlight the potential of melatonin as a therapeutic strategy for mitigating the adverse effects of environmental pollutants on oxidative stress and subsequent developmental outcomes. Overall, our study contributes to the growing body of evidence supporting the protective role of melatonin against developmental toxicity and oxidative damage

induced by environmental contaminants such as BPA and BPS[395,396]. Further research is warranted to elucidate the underlying mechanisms of melatonin-mediated protection and to evaluate its efficacy in mitigating the adverse effects of other environmental pollutants. The insights gained from this study may have important implications for the development of novel therapeutic interventions to safeguard against the detrimental effects of environmental exposures during critical periods of development.

5. Conclusion

In this study, we found that exposure to BPA, BPS alone, or in combination with MP, resulted in significant damage to the liver and intestines of adult zebrafish, leading to notable oxidative stress and cellular apoptosis. Moreover, the offspring zebrafish, not directly exposed to the compounds, also exhibited significant developmental toxicity and oxidative damage, demonstrating the transgenerational toxicity of BPA and BPS in zebrafish. Additionally, we discovered that partial restoration of oxidative damage was achieved with melatonin treatment. These findings highlight the importance of comprehensive assessment and management of environmental health risks to support the conservation of species in aquatic environments.

Chapter 5

Reproductive toxicity of parental co-exposure of bisphenol compounds and microplastics on adult zebrafish: multi-omics investigations on offspring

Adapted from:
Xue, M., Jia, M., Qin, Y., Francis, F., & Gu, X. (2024). Toxicity of parental co-exposure of microplastic and bisphenol compounds on adult zebrafish: Multi-omics investigations on offspring. Science of The Total Environment, 17678.
https://doi.org/10.1016/j.scitotenv.2024.176897

Abstract

In recent years, the widespread use of bisphenol compounds and microplastics (MP) have attracted attention due to their harmful effects. Here, individual and combined effects of MP and bisphenol compounds, were assessed on adult zebrafish after co-exposure of bisphenol A (BPA) or bisphenol S (BPS) and 25μm polyethylene MP. Impacts on their offspring (the F1 generation) were also investigated. The reproductive toxicity in adult zebrafish impacted exerted by bisphenol compounds were aggravated by the co-presence of MP. Transcriptomics and metabolomics further showed single or co-exposure of bisphenol compounds and MP could together regulate apoptosis, calcium signaling pathway and glycerophospholipid signaling pathways. Our results also showed the different toxicity mechanisms on transcriptional and metabolic profiles in the combination effects of bisphenol compounds and MP. The co-exposure of BPA and MP predominantly influenced neurotoxicity via the MAPK signaling pathway and voltage-dependent calcium channels, whereas the co-exposure of BPS and MP principally affected visual development through phototransduction and retinol metabolism. The co-exposure of BPA and MP, as well as BPS and MP, specifically regulate lipid metabolism and carbohydrate metabolism in zebrafish offspring, respectively. Overall, this study provided a deep understanding of the toxicity differences between co-exposure and single exposure of bisphenol compound and MP in zebrafish, as well as the transgenerational effects and potential molecular mechanisms of bisphenol compounds and MP in zebrafish offspring.

Keywords: Bisphenol compounds, Microplastic, Co-exposure, Transcriptomic, Metabolomics

1. Introduction

Microplastics (MP), which are becoming a new type and a fast-growing environmental pollutant, have raised high priority concerns[397,398]. At the second United Nations Environmental Conference in 2015, MP pollution was identified as the second most critical scientific issue in the field of environmental and ecological science[399]. MP have recently been found worldwide in marine systems[85,400–402], sediments[403–405], surface waters[406–409] and drinking waters[410,411]. The presence of MP has also been reported in aquatic organisms[412]. Aquatic environment is the focus of MP. For example, in the surface waters of the Northwestern Pacific Ocean, MP concentrations are ranging from 640 to 42,000 items/km^2[,85]. And Dusaucy et al. (2021) reported aquatic environment, such as rivers, lakes and oceans, have frequently observed high concentration of MP (0.27 to 34,000 particles/m^3)[41]. Remarkably, MP have also been found in human feces[413] and in human blood[414], indicating potential toxic effects to human health.

The negative and irreversible impacts of MP have already been demonstrated on animal growth and oxidative stress, as well as energy and lipid metabolism disorders and neurotoxicity[17,130,415,416]. Moreover, due to their large specific surface area and strong affinity to hydrophobic chemicals, MP could adsorb other surrounding contaminants and serve as concentrator and transporter for toxic substances[417–420].

Bisphenol compounds (BPs) are endocrine disrupting chemicals (EDCs), of which bisphenol A (BPA) is the most common that has been used for many years[421]. BPA were present in almost all environment media, including sediment[225], surface waters including lakes[422,423] and drinking water[424]. Currently, BPA was also be detected in aquatic organisms and even humans[222,425,426]. Several studies have confirmed the BPA and its substitutes frequently detected in river, lake and sea water, with their concentrations ranged from 0.00001 to 85.5μg/L[218,219,228].

As a typical endocrine disruptor, BPA has been associated with various pathologies, such as metabolic diseases nervous and immune system disorders, reproductive toxicity and cardiovascular diseases[427–430]. Because of its toxic and endocrine-disrupting properties, its use has been limited and banned in many countries[431]. These restrictions had led to manufacturers to use substitutes such as bisphenol F (BPF), bisphenol S (BPS) and bisphenol B (BPB)[32]. BPS has become one of the main alternatives for production of BPA-free products[290]. However, some studies reported that substitutes for BPA were continually to be detected in various environmental media[32], and confirmed that substitutes exert similar toxicities as BPA[432,433].

In natural environments, MP could serve as vectors and coexist with other contaminants[434]. The interactions between MP and other contaminants could affect the environmental behavior of the latter, leading to combined effects on organisms[435]. For instance, Li et al. (2021) discovered that polyethylene MP could increase the

bioavailability of metals after their co-exposure[436]. Granby et al. (2018) highlighted that MP could potentiate the adverse effect of polychlorinated biphenyls contaminants[419]. Some studies have also reported the impacts of co-exposure to MP and BPs on organisms. Chen et al. (2017) demonstrated that the presence of nano plastics could increase BPA bioavailability and cause neurotoxicity in adult zebrafish[308]. Mu et al. (2022) found that MP would enhance the toxicity toward fish larvae when co-exposed with BPs[33]. Then, it is urgent to develop further investigations on the harmfulness between MP and other pollutants.

Reproductive dysfunction is one of the most significant impacts of the estrogenic effects of BPs[310]. Zebrafish possess unique advantages as a powerful model for testing endocrine disruptors and the study of environmental toxicity[243]. The endocrine system could be disrupted by the hypothalamic-pituitary-gonadal-liver (HPGL) axis and ultimately results in reproductive abnormalities in zebrafish[437,438]. Currently, effects of environmental pollutants on aquatic animals are mainly focused on the single exposure[439], few data's have been reported on the combined toxic effects under the coexistence of MP and BPs[30,33]. Most importantly, these reports only examine alterations in a single generation following either single or combined exposure. It has been reported that the adverse effects of BPs could be transferred from mother to foetus[440].

Then, the purpose of our study is to investigate the combined toxic effects of BPA and BPS, with or without the co-presence of MP, and integrated omics techniques, namely transcriptomics and metabolomics to clarify the underlying mechanisms of the effects on the offspring (F1 generation) after parental exposure on adult zebrafish. Moreover, to the best of our knowledge, this is one of very first studies that link transcriptomes and metabolomes on F1generation with toxic effect on adult zebrafish after parental exposure of BPA/S and MP or in combination. This study provides new insights into reproductive toxicity induced by BPs and MP in zebrafish, as well as the mechanisms of transgenerational toxicity. These findings have potential implications for both human and animal health.

2. Materials and method

2.1. Chemicals

The standard of BPA (CAS:80-05-7) (purity 99.8%) and BPS (CAS:80-09-1) (purity 99%) were purchased from Aladdin Biochemical Technology Co., Ltd (Shanghai, China). Polyethylene MP were selected as representative MP in this study with particle size of 25μm were purchased from Zhichuan Technology Co., LTD (Jiangsu, China). Methanol and acetonitrile were purchased from Thermo Fisher Scientific Inc (Shanghai, China). All reagents were HPLC grade. The stock solutions of BPs (400μg/L) were

prepared by dissolving appropriate amount of each standard in methanol. All solutions were stored at -20°C until use.

2.2 Animals and treatment

Adult zebrafish (strain AB) were obtained from the aquarium department of Hongdagaofeng, and have been continuously reared in the laboratory for two weeks before the exposure tests (14h light/10h dark cycle, 25.0 ± 1.0°C). During the acclimation period, fishes were fed two times daily. During the acclimatization, the rearing water was renewed every three days.

2.3 Exposure test

The toxicity test with adults was conducted with reference to the standard protocols of OECD guidelines (OECD, 2019). Adult zebrafish were randomly selected and exposed to different treatments: control (Ctr) group (dechlorinated tap water + 0.002% methanol), BPA group (100μg/L of BPA), BPS group (100μg/L of BPS), MP group (100μg/L of MP), MA group (100μg/L of BPA +100μg/L of MP) and MS group (100μg/L of BPS +100μg/L of MP). The control and exposure groups received 0.002% (v/v) methanol. The concentration of exposure (100μg/L) was based on the environmentally relevant concentrations and could induce clearly effects and identify possible mechanisms of toxicity[336,441].

Five liters glass beakers containing 4 L of liquid and 10 adult fishes were set for each treatment with six replicates. The solutions were changed every 3 days to ensure stability of chemical substance concentration. The exposure period was 35 days, as this duration is to more accurately reflect real environmental conditions and to better assess the long-term effects and biological responses of low concentrations of bisphenol compounds on aquatic organisms. During the experiment, external conditions, including temperature, humidity and light cycle, were consistent with the domestic environment.

2.4 Sampling collection

Adult zebrafish. After 35d exposure, five 24h starved individuals per replicate were randomly collected and to be anaesthetized in MS 222 (Tricaine, Sigma-Aldrich). The blood samples were collected from the caudal vein and centrifuged at 3000g, 15 min. The separated serum was extracted on ice and stored at −80 °C for later analysis. Muscle, brain, gill, gonad, liver and intestinal tissues were removed quickly from ice. All samples were stored at −80 °C until extraction and analysis.

Embryo. After exposure of 35d, 2 fish females and 2 males were randomly selected from different treatment groups and paired in spawning boxes. Fertilized eggs were

collected and reared in incubator at 28℃. After 72h, the F1 offspring were collected and stored at −80 °C for the following analysis.

2.5 Histological analysis

For histopathological observation, gonads from female fish were fixed in 4% paraformaldehyde stationary solution for 24h. The fixed ovaries were dehydrated in graded ethanol and xylene, and embedded in paraffin to be sectioned at 4μm. Then, the gonad tissues were stained with hematoxylin-eosin (HE) to measure the pathological changes.

2.6 Measurement of sex hormones and vitellogenin

The estradiol (E2), testosterone (T), 11-keto testosterone (11-KT) and vitellogenin (VTG) were measured by competitive enzyme-linked immunosorbent assay (ELISA) using commercial kits (Jiangsu Meimian Industrial Co., Ltd) according to the manufacturer's instructions. All biological samples were processed in triplicate.

2.7 Gene expression analysis

To analyze the reproductive system after treatment on adult zebrafish, 17 genes related to the HPGL axis were tested. Two gonadotropin-releasing hormone genes (*gnrh2, gnrh3*), two gonadotropin-releasing hormone receptor genes (*gnrhr2, gnrhr3*), the luteinizing hormone beta gene (*lhβ*), the luteinizing hormone receptor gene (*lhr*), the follicle stimulating hormone beta gene (*fshβ*), the follicle-stimulating hormone receptor gene (*fshr*), hydroxymethylglutaryl CoA reductase genes (*hmgr*), steroidogenic acute regulatory protein genes (*star*), three cytochrome P450 genes (*cyp11a, cyp19a, cyp19b*), two estrogen receptor genes (*erα, erβ*), the androgen receptor gene (*ar*), and the vitellogenin 1 gene (*vtg1*). As an internal control gene, β-actin was used to normalize the expression profile. All primer sequences for these genes are listed in Supplementary Table S5-1. We used TRIzol reagent (Tiangen Biochemical Technology, China) to extract the total RNA from the brain, gonads and liver (n=5). cDNA synthesis and SYBR Green real-time PCR were performed using commercial kits (Tiangen Biochemical Technology, China). Three biological replicates were used for each treatment.

2.8 Transcriptomic analysis

Total RNA was extracted using the TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. RNA purity and quantification were evaluated using the NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). RNA integrity was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Then the libraries were constructed using VAHTS Universal V6 RNA-seq

Library Prep Kit according to the manufacturer's instructions. The transcriptome sequencing and analysis were conducted by OE Biotech Co., Ltd. (Shanghai, China). The following detailed RNA sequencing and differentially expressed genes analysis are described in Supplementary Text S5-1.

2.9 Gene expression analysis in F1 offspring

To verify the RNA-seq results, the differentially expressed genes (DEGs) identified in different treatments were measured by RT-PCR. The RNA samples were prepared in triplicate in each treatment for RT-PCR. The gene transcriptions were analyzed using the $2^{-\Delta\Delta CT}$ method with *β-actin* as the housekeeping gene. The primer sequences were listed in Supplementary Table S5-2.

2.10 Metabolomics analysis

The detailed process of metabolites extraction are described in Supplementary Text S5-2. An ultrahigh performance liquid chromatography system (UHPLC) coupled to a QExactive Focus Hybrid Quadrupole-Orbitrap Mass Spectrometer (QE Orbitrap MS) in both positive and negative ionization modes (Thermo Fisher Scientific, China), with an electrospray ionization (ESI) source was applied to the data acquisition. A Waters UPLC HSS T3 column (100 mm×2.1 mm, 1.8um) was used for characteristic peak separation. The following detailed instrument parameters are listed in Supplementary Table S5-3. Detailed data analysis for metabolomics is described in Supplementary Text S5-3.

2.11 Statistical analysis

All results data were expressed as mean ± standard errors and obtained data were analyzed and visualized by GraphPad Prism 7.0. Statistical significance between the exposure and control groups was determined using an independent t-test. Differences with $p < 0.05$ were considered statistically significant.

3. Results

3.1 Histological analysis

The structure of the ovaries in the control group was intact, with oocytes filled with red yolk granules and an intact external follicular membrane (Figure 5-1A). Pathological changes of varying degrees were observed in other treatment groups. Within MP, BPA, BPS groups, there was a loss of contact between the oocyte membrane and the follicular cell layer, a degradation of the yolk cells as well as cell lysis (Figure 5-1B, C, E). In the combined exposure treatment, MA and MS groups (Figure 5-1D, F), ovarian

pathological damages continued to worsen, with oocyte degradation becoming more severe and a noticeable reduction in the number of mature, developed oocytes.

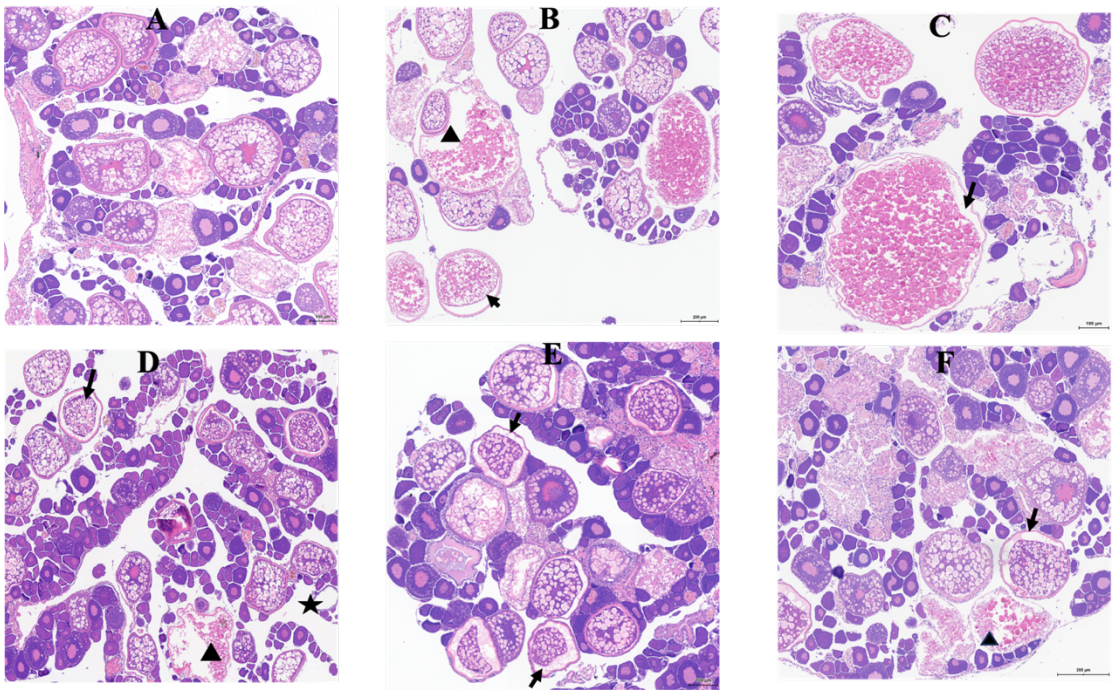

Figure 5-1. Histological examinations in female ovary section. A: Control group. B: MP group. C: BPA group. D: MA group. E: BPS group. F: MS group. **Arrows represent:** the loss of contacts between the oocyte cell membranes and the follicular cell layer. **Stars represent:** the vacuolation of the gonadosomatic tissue. **Triangles represent** degenerating vitellogenic oocytes.

3.2 Sex hormone and VTG measurement

For female fish, E2 levels were significantly decreased in BPA, MA and MS groups (6.36pmol/L, 6.93pmol/L, 6.49pmol/L) compared to Ctr group (9.62pmol/L), while there were no significant differences in BPS (8.64pmol/L) and MP groups (9.89pmol/L) (Figure 5-2A). The levels of changes in VTG were similar to that of E2 (Figure 5-2D). Compared to those in the Ctr group (16.29nmol/L), T levels presented significantly decreasing trend excepting for BPS treatment (MP:13.86nmol/L; BPA:10.85nmol/L; MA:9.27nmol/L; MS:10.38nmol/L) (Figure 5-2B). In all groups, 11-KT showed significantly decreased trend. The level of 11-KT in the Ctr group was 17.62pg/mL; in MP, BPA, MA, BPS and MS groups, this value decreased to 13.18pg/mL, 11.15pg/mL, 12.30pg/mL, 14.92pg/mL, 10.98pg/mL, respectively. (Figure 5-2C). Moreover, the BPS group and the co-exposure with MP group exhibited significant differences in the levels of four indicators, with a more pronounced decrease levels in co-exposure groups. This indicated that the toxicity of MP in combination with BPS was stronger than the toxicity of BPS exposure alone. Similarly, there were significant differences in all tested parameters between the BPA and BPS treatment groups, indicated that BPA had

stronger toxicity than BPS in zebrafish. An intriguing finding was that the changes in hormone levels were less pronounced in male fish compared to female fish, suggesting that the latter were more sensitive to bisphenol compounds than males.

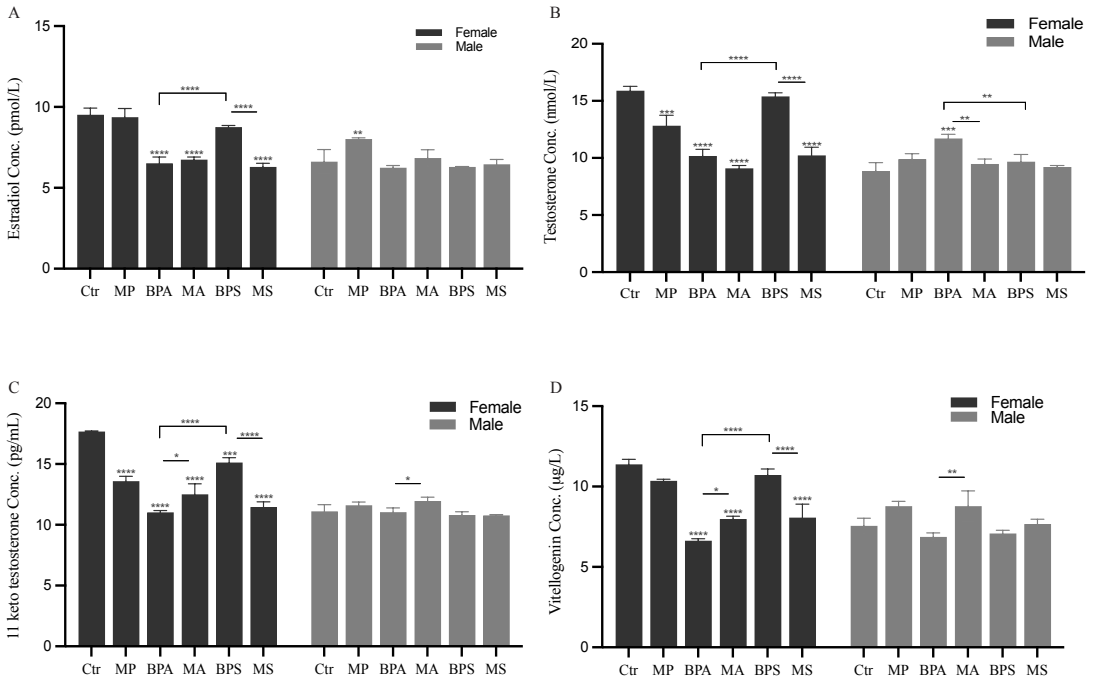

Figure 5-2. A: Estradiol (E2), B: testosterone (T), C: 11-keto testosterone (11-KT) and D: vitellogenin (VTG) levels in male and female fish. The asterisk symbols denote significant differences between different groups. The data were analyzed by one-way ANOVA test (*p < 0.05, **p < 0.01, ***p < 0.001 and ****p <0.0001).

3.3 Gene expression analyses related of HPGL axis

Expression levels of *gnrh2* and *gnrh3* in BPA and MA groups were increased significantly (Figure 5-3). However, the mRNA levels of *gnrhr2* and *gnrhr3*, excepting for MP group, were significantly decreased in all other groups. Among the expressions of *fshr* and *fshβ*, only MA group showed a significant increase. Conversely, in the expression of *hmgr*, only MA group demonstrated a significant decrease. There had a downregulation in the mRNA levels of *cyp11a*, *lhr*, *erα* and *vtg*. The level of *lhβ* and *erβ* were upregulated, but within the expression of *erβ*, only the upregulation in the MA group was significant. It's worth noting that the expression of *cyp19a*, *cyp19b* and *star*

in the MA group showed a significantly different trend compared to the other treatment groups.

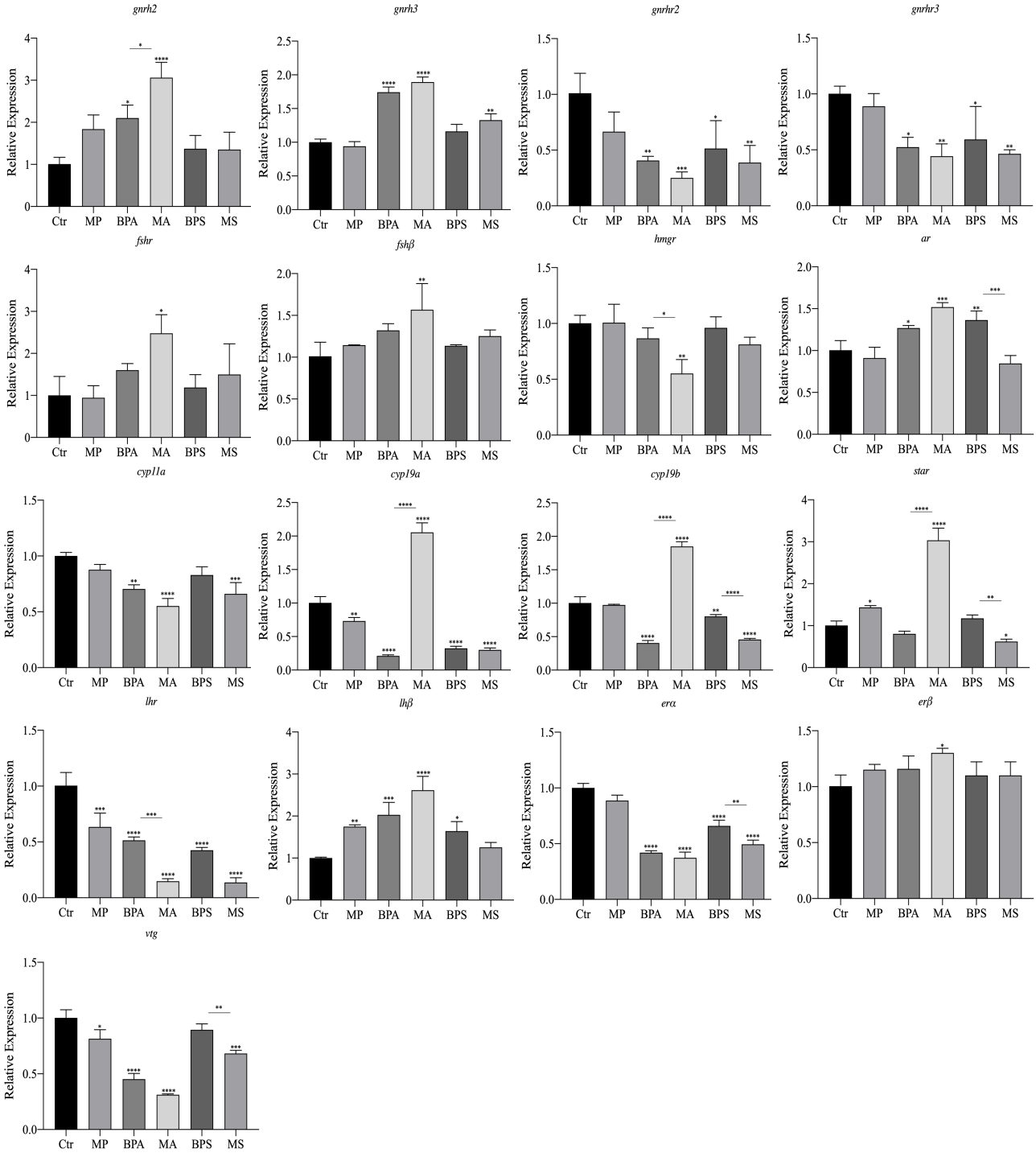

Figure 5-3. Relative transcript levels of hypothalamic-pituitary-gonadal-liver (HPGL) axis genes of adult female zebrafish. The data were analyzed by ANOVA test (*p < 0.05, **p < 0.01, ***p < 0.001 and ****p <0.0001)

3.4 Transcriptomic analysis on F1 offspring

To get more specific understanding of the responsible for the toxicity of parental BPA/S and MP exposure to F1 progeny, the control and five experimental groups (MP, BPA, BPS, MA and MS) on F1 offspring were analyzed for transcriptomic analysis. Exposure to MP resulted in total 981 DEGs in zebrafish embryos, among these DEGs, 576 genes were up-regulated and 405 genes were down-regulated. RNA-seq identified 753 DEGs after BPA exposure, 442 genes were up-regulated and 311 down-regulated. Total 1064 DEGs were identified after MA treatment, including 721 up-regulated DEGs and 343 down-regulated DEGs. In the BPS group, 363 DEGs were identified, including 228 up-regulated DEGs and 135 down-regulated DEGs. MS exposures resulted in 1288 DEGs, with 895 up-regulated DEGs and 393 down-regulated DEGs (Figure 5-4A). The number of DEGs in co-exposure groups (MA and MS group) were higher than that in single exposure groups (BPA, BPS and MP group).

The significantly enriched GO (gene ontology) terms of DEGs for biological process, cellular component and molecular function after MP, BPA, MA, BPS and MS exposures are shown in Figure S5-1. Further KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analysis of the top 20 significantly enriched KEGG pathways in 5 treatment groups were shown in Figure S5-2. Next, 39 shared differential genes were found among five treatment groups (Figure 5-4B). We also investigated the KEGG enrichment analysis for 39 common dysregulated genes. These shared genes were mainly enriched in apoptosis, ABC transporters and calcium signaling pathway (Figure 5-4C) and were analyzed and visualized using a heatmap (Figure 5-4D). In apoptosis pathway, the *fosaa* were upregulated in all five treatments, while the *si:dkey-269i1.3 (zgc:174154)* was significantly upregulated only in BPS group and generally downregulated in the other treatment groups. The downregulation gene of *abcg2d* and the upregulation gene of *abcc8b* have been assigned to the regulation of ABC transporters. The three dysregulation genes of *mcoln3a*, *LOC569566* and *camk1ga* among five treatments have been categorized under the regulation of calcium signaling pathway.

In our present study, we mainly focused on the differential impacts on offspring resulting from parental co-exposure and single exposure to BPs and MP. Then, we also examined the differential genes between co-exposure groups and single exposure groups. We found that there had 603 unique differential genes in co-exposure MA group compared to BPA and MP groups (BPA vs. MP vs. MA) (Figure 5-5A). And 725 unique differential genes were identified in MS group compared to BPS and MS groups (BPS vs. MP vs. MS) (Figure 5-5B). Heatmaps of these unique differential genes in co-exposure groups were showed in Figure S5-3A and Figure S5-3B, respectively. KEGG enrichment analysis were carried out to better understand which pathways were affected after co-exposure. The unique differential genes in MA group were mainly enriched in

neuroactive ligand-receptor interaction and MAPK (mitogen-activated protein kinase) signaling pathway (Figure 5-5C).

The expression levels of unique differential genes in neuroactive ligand–receptor interaction pathway were showed in Figure 5-6A, while those in the MAPK signaling pathway were depicted in Figure 5-6B. Phototransduction and retinol metabolism were also identified by KEGG analysis in MS group (Figure 5-5D). The expression levels of unique differential genes in these pathways were displayed in Figure 5-6C and 6D.

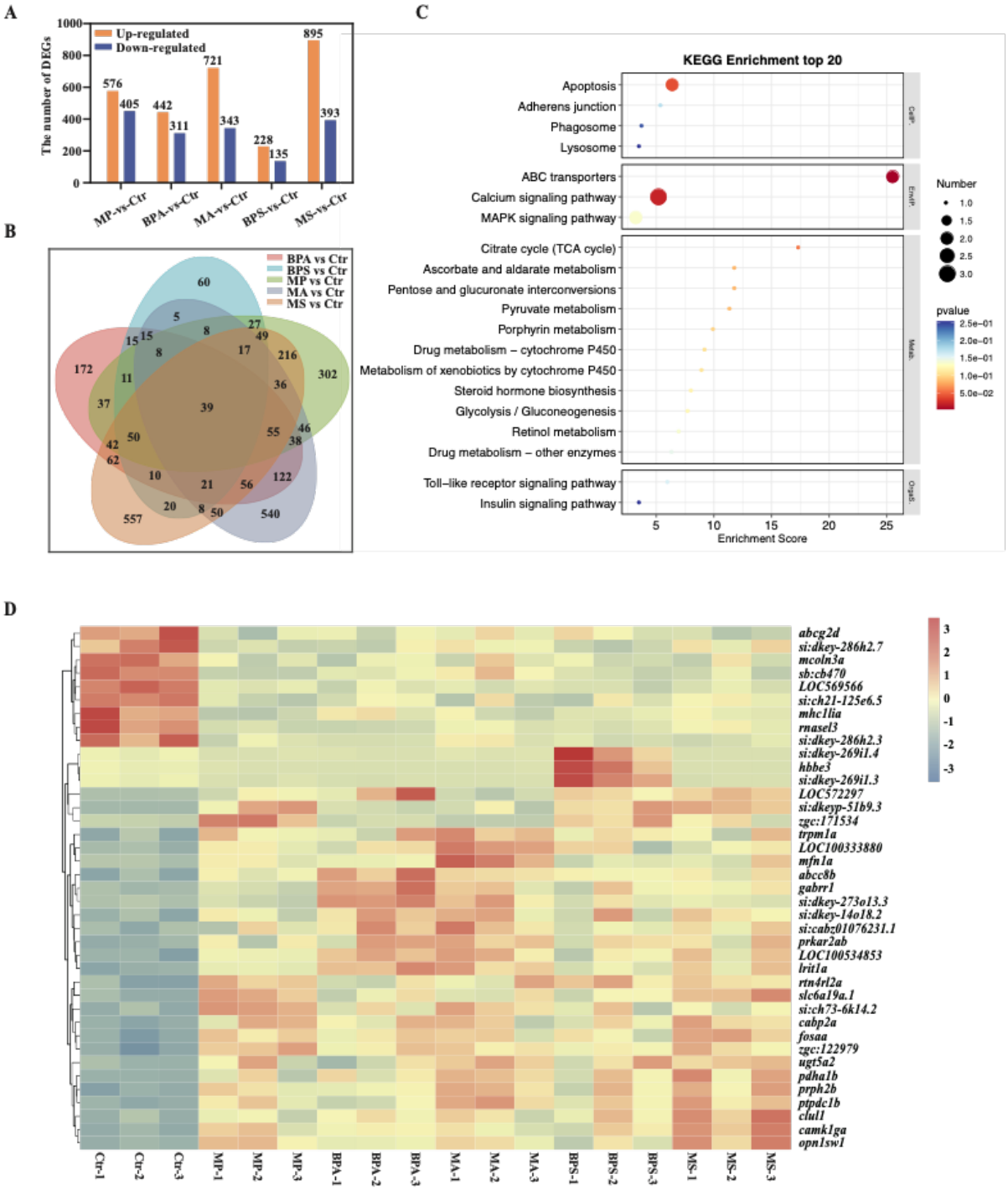

Figure 5-4. Transcriptomic of the DEGs in embryos after parental exposure of different treatment. A: The numbers of DEGs in embryos after parental exposure (q <0.05& log2FoldChange>1). B: A venn diagram showing the shared or unique genes among exposure groups. C: KEGG pathway analysis of 39 shared DEGs. Y-axis represents pathways and X-axis represents the enrichment score. The color and size of each bubble represent the enrichment significance and the number of genes enriched in the pathway, respectively. D: Heatmap clustering for the 39 shared DEGs between 5 treatments and control. Red represents up-regulation and blue represents down-regulation.

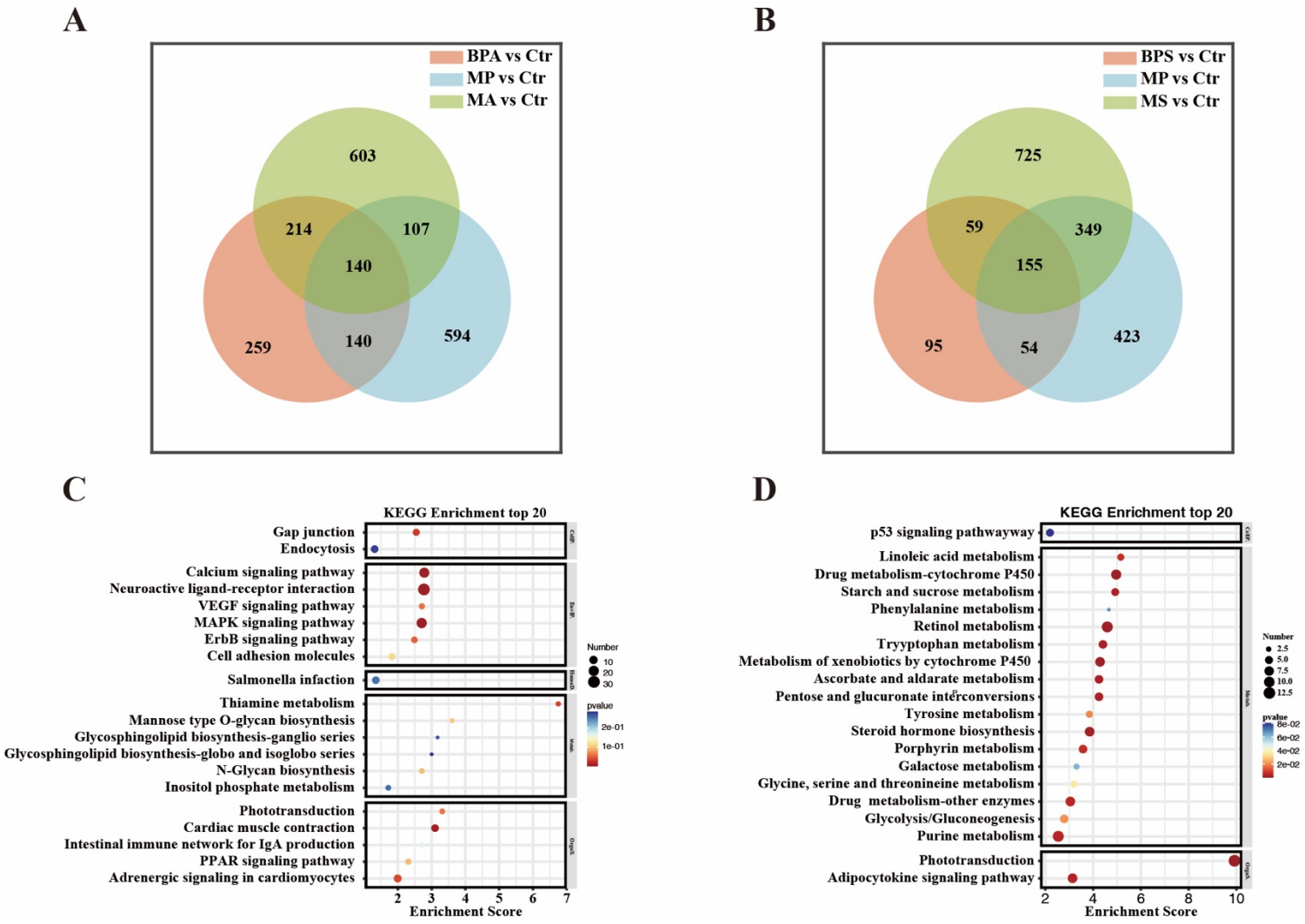

Figure 5-5. A: The venn diagram of DEGs in BPA, MP and MA treatments. B: The venn diagram of DEGs in BPS, MP and MS treatments. C: The top 20 significantly enriched KEGG pathways of 603 unique differential genes in MA group. D: The top 20 significantly enriched KEGG pathways of 725 unique differential genes in MS group.

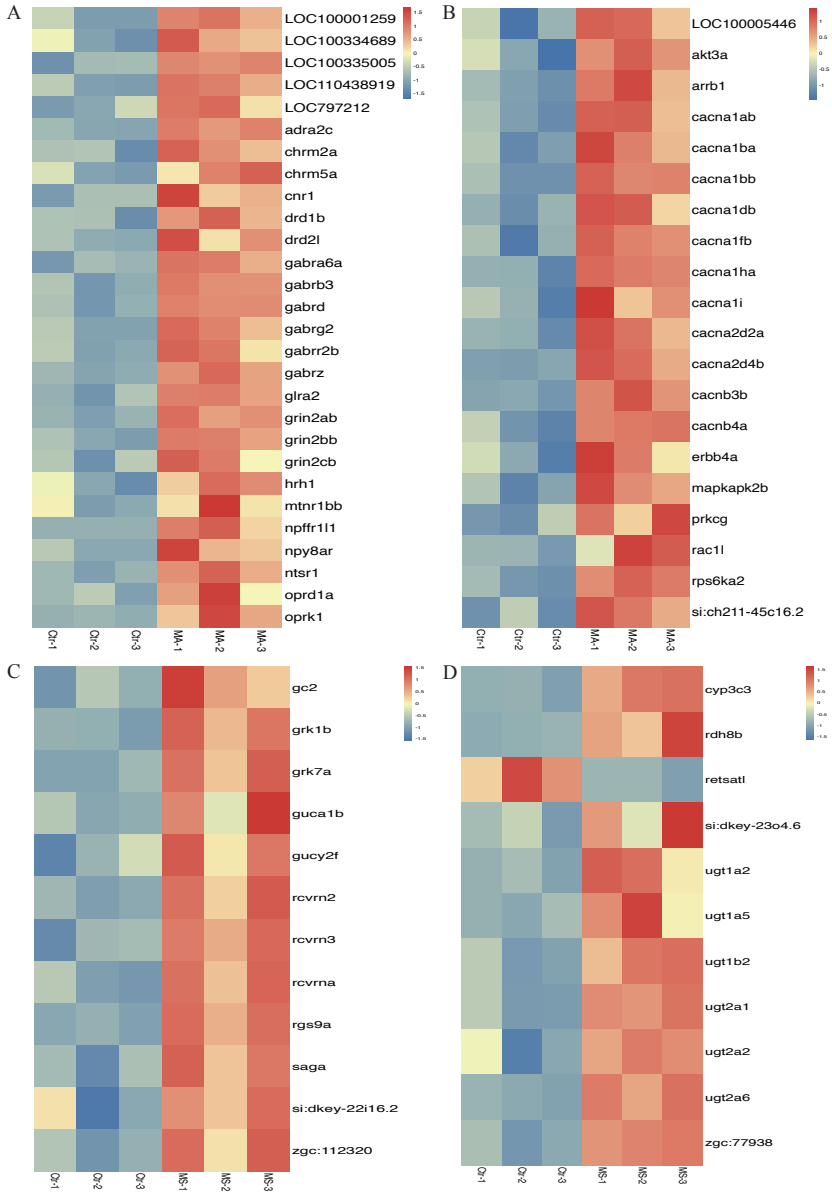

Figure 5-6. A: Transcriptome heatmap of unique differential genes related to neuroactive ligand–receptor interaction pathway in MA group. B: Transcriptome heatmap of unique differential genes related to MAPK signaling pathway in MA group. C: Transcriptome heatmap of unique differential genes related to phototransduction pathway in MS group. D: Transcriptome heatmap of unique differential genes related to retinol metabolism pathway in MS group.

3.5 RT-qPCR

The results of RNA-Seq were verified via RT-qPCR. We selected 9 differential genes (*fosaa*, *prph2b*, *abcc8b, pdha1b, opn1sw1, prkar2ab, mfn1a, clul1* and *hbbe3*) from different enrichment pathways according to the function and KEGG analysis. The

relative mRNA expression levels of genes were basically consistent with those of transcriptome analysis results (Figure S5-4).

3.6 Metabolomics analysis on F1 offspring

Score plot of both PCA (Principal Component Analysis) (Figure S5-5) and OPLS-DA (Orthogonal Partial Least Squares-Discriminant Analysis) (Figure S5-6) of zebrafish embryos data indicated the good separations of experimental treatments. There were 245, 169, 199, 101 and 133 significantly changed metabolites (VIP>1 and p-value<0.05) (VIP: Variable important in projection) that had been identified in the MP, BPA, MA, BPS and MS group, respectively. Among them, 202, 129, 168, 79 and 95 differential metabolites were up-regulated, and 43, 40, 31, 22 and 38 differential metabolites were down-regulated, respectively (Figure 5-7A). KEGG pathway enrichment analysis of the differential metabolites in the different treatment groups were shown in Figure S5-7. In Venn diagram (Figure 5-7B), 17 shared differential metabolites were found in five exposure groups. These shared differential metabolites were shown to be highly associated with the glycerophospholipid metabolism by KEGG enrichment analysis (Figure 5-7C). A heatmap illustrating these shared differential metabolites changes among five different treatments (Figure 5-7D). Then, 58 and 52 unique differential metabolites in MA (BPA vs. MP vs. MA) and MS groups (BPS vs. MP vs. MS) respectively were found between single and co-exposures (Figure 5-8A and 8B).

In order to clarify the role of these unique metabolites in the mechanism of co-exposure of BPs and MP triggered toxicity, the KEGG enrichment analysis were carried out. The unique differential metabolites of MA group were mainly enriched in arachidonic acid metabolism (Figure 5-8C). Unique dysregulated metabolites in MS group were enriched in pyruvate metabolism and glycolysis/gluconeogenesis metabolism pathway (Figure 5-8D). Additionally, the heatmap of the expression pattern of these unique dysregulated metabolites in co-exposure groups were showed in Figure S5-8A and S5-8B.

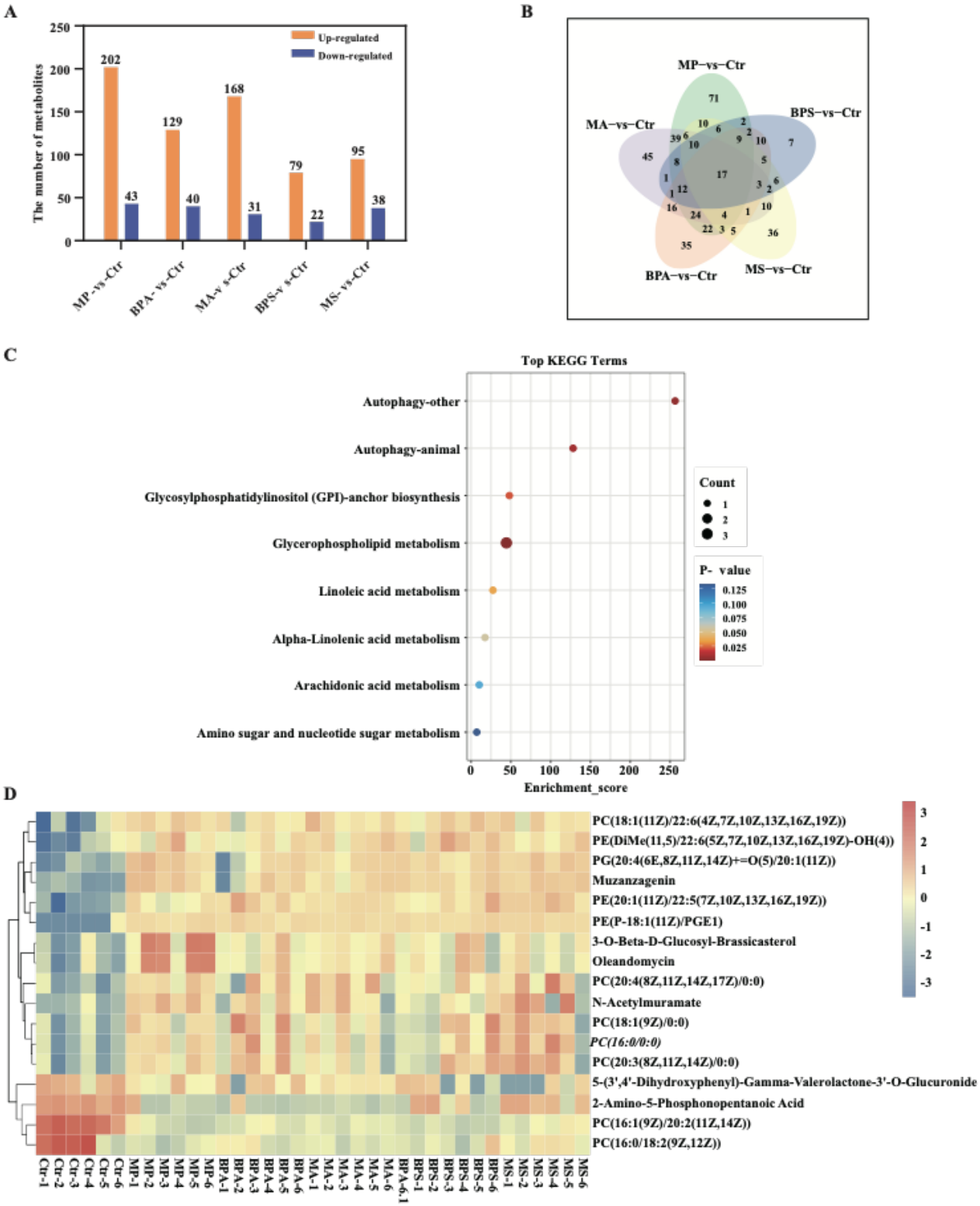

Figure 5-7. Metabolomics of differential metabolites in embryos after parental exposure of different treatment. A: The numbers of differential metabolites in embryos after parental exposure. B: A venn diagram showing the shared or unique genes among exposure groups. C: KEGG pathway analysis of 17 shared differential metabolites. D: Heatmap clustering for the 17 shared differential metabolites between 5 treatments and control. Red represents up-regulation and blue represents down-regulation.

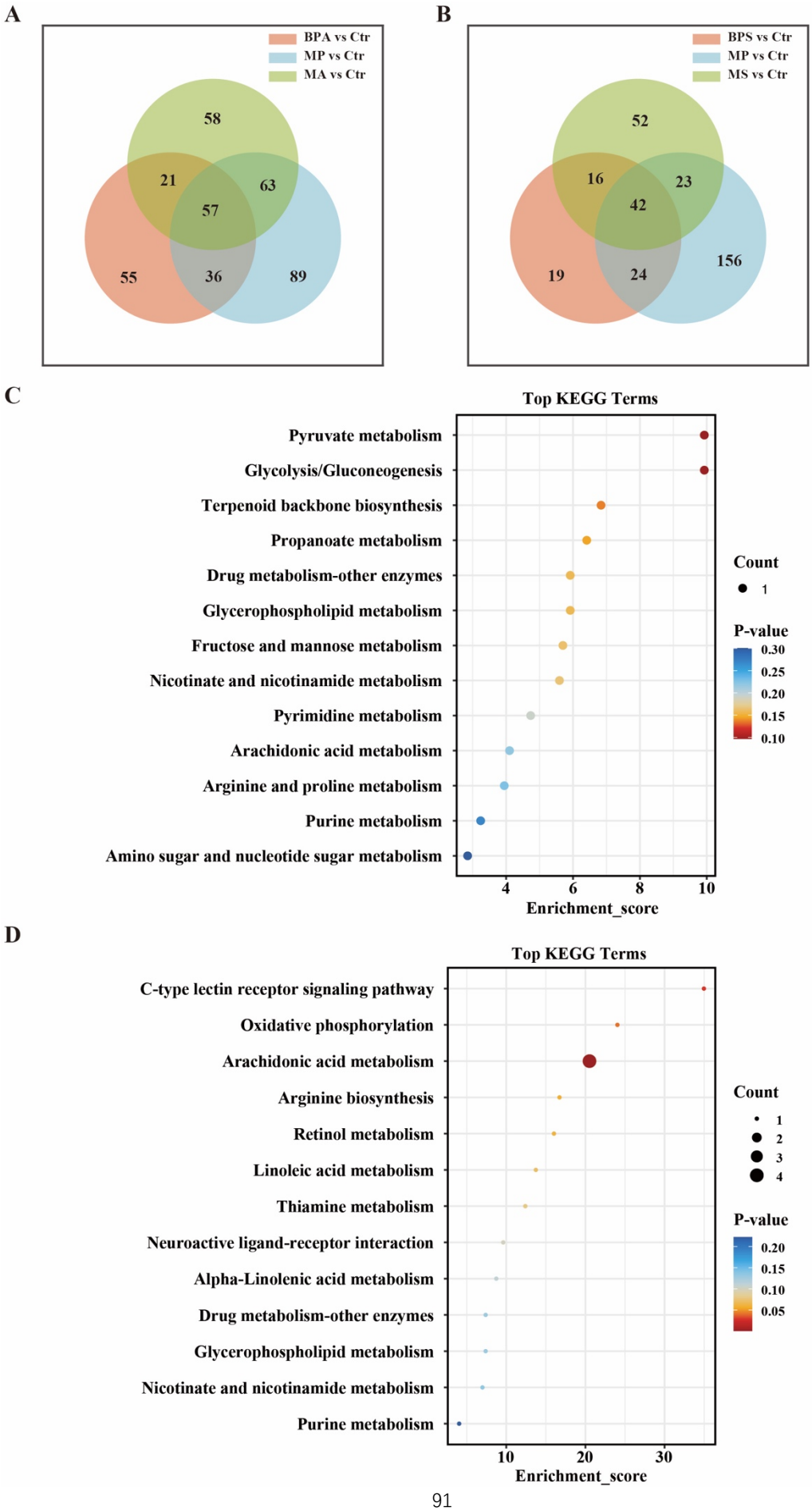
A
BPA vs Ctr
MP vs Ctr
MA vs Ctr
58
21
63
57
55
36
89
B
BPS vs Ctr
MP vs Ctr
MS vs Ctr
52
16
23
42
19
24
156
C
Top KEGG Terms
Pyruvate metabolism
Glycolysis/Gluconeogenesis
Terpenoid backbone biosynthesis
Propanoate metabolism
Drug metabolism-other enzymes
Glycerophospholipid metabolism
Fructose and mannose metabolism
Nicotinate and nicotinamide metabolism
Pyrimidine metabolism
Arachidonic acid metabolism
Arginine and proline metabolism
Purine metabolism
Amino sugar and nucleotide sugar metabolism
4
6
8
10
Enrichment_score
Count
1
P-value
0.30
0.25
0.20
0.15
0.10
D
Top KEGG Terms
C-type lectin receptor signaling pathway
Oxidative phosphorylation
Arachidonic acid metabolism
Arginine biosynthesis
Retinol metabolism
Linoleic acid metabolism
Thiamine metabolism
Neuroactive ligand-receptor interaction
Alpha-Linolenic acid metabolism
Drug metabolism-other enzymes
Glycerophospholipid metabolism
Nicotinate and nicotinamide metabolism
Purine metabolism
10
20
30
Enrichment_score
Count
1
2
3
4
P-value
0.20
0.15
0.10
0.05

Figure 5-8. Metabolomics analysis of the changed metabolites in embryos after different treatment. A: The venn diagram of differential metabolites in BPA, MP and MA treatments. B: The venn diagram of differential metabolites in BPS, MP and MS treatments. C: The top 20 significantly enriched KEGG pathways of 58 unique differential metabolites in MA group. D: The top 20 significantly enriched KEGG pathways of 52 unique differential metabolites in MS group.

4. Discussion

In this study, we assessed the co-exposure of BPs and polyethylene MP at environmentally relevant levels on the reproductive toxic of adult zebrafish and explored the molecular mechanisms of transgenerational effects on their offspring.

4.1 Effects on ovary histology

The adverse effects of BPs on mammalian oocytes have been demonstrated such as leading to a cessation of meiosis in mouse oocytes and modifying the gene expression pattern in human fetal oocytes[442,443]. Not limited in mammals, the negative impacts of BPs on fish ovary have also been reported. Chronic BPA exposure increased the oxidative stress and membrane damage in zebrafish ovary[253] and promoted cell death, poor oocyte quality and attenuation of meiotic maturation in carp *L. bata*[358]. In the present study, compared to the control, various damages could be observed in different treatments. The oocytes exposed to MA and MS groups were smaller in diameter and less mature compared to single exposure groups. This demonstrated that co-exposure of BPA/S and MP could inhibit the development and maturation of ovaries that indicating that co-exposure could pose a stronger threat to zebrafish.

4.2 Effects on the transcriptional expression of genes in HPGL axis and hormone level

The reproductive process in fish is regulated by the coordinated interaction between steroid hormones on the HPGL axis and steroidogenesis[444,445]. BPA and its alternatives exert effect on the HPGL axis, which could control regulatory system on ovarian maturation in fish[446,447]. Therefore, we investigated alterations in the expression of hormone-related genes and fluctuations in endocrine hormone levels.

The dysfunctional GnRH (Gonadotropin-Releasing Hormone) system had been identified the responsible for absence of ovulation and spawning in aquaculture species[448]. Like mammals, GnRH is also involved in the regulatory processes of reproduction in teleost, encompassing aspects such as spawning activity[449] and the development of oocytes[450]. In teleost fish, multiple studies have documented that the two variants of GnRH (*gnrh2* and *gnrh3*) could stimulate the secretion of FSH (follicle-

stimulating hormone) and LH (luteinizing hormone) in the pituitary gland[448]. In this study, we observed significant upregulations of *gnrh2* and *gnrh3* in BPA and MA group, alignment with the observed trend in the changes of *lhβ*, which corroborates the above statement. That means BPA exposure could more directly affect the GnRH system than BPS exposure in adult zebrafish. However, their receptors (GnRHRs) were disrupted, *gnrhr2* and *gnrhr3* showed significant downregulations except MP group, contrasting with the *gnrh2* and *gnrh3* trend, indicating that the equilibrium between GnRHs and their receptors[451]. This disruption could lead to an imbalance of sex hormones in fish[452].

The concentration levels of E2 and T hormones have been commonly used as key biomarkers for reproduction in zebrafish[243]. *cyp19a* and *cyp19b* play a crucial role in sex differentiation and gonad development, converting T to E2[453,454]. The mRNA level of *cyp19a* and *cyp19b* were generally downregulated in this study which could supported the changes in the two sex hormones. However, in MA treatment group, the expression levels of *cyp19a* and *cyp19b* surprisingly increased by several fold, and the expression of *star* followed the same pattern. This might be due to the co-exposure to BPA and MP, which exerted an overly strong toxic effect on zebrafish, and zebrafish self-repaired and self-adjusted under stress, leading to reverse changes in the genes. Moreover, E2 synthesized by the gonads, induced the liver to produce VTG, facilitating the growth and maturation of oocytes[455]. The findings of this study corroborated this understanding. The noted decrease in *vtg* expression in zebrafish can be considered a result of reduced E2 levels. However, alterations in both *vtg* and E2 were not pronounced within the single exposure of BPS, suggesting that the hormonal responses in zebrafish exhibit greater sensitivity to BPA exposure and co-exposure.

Many studies have demonstrated that BPA and its alternatives could as agonist or antagonist to disturb physiological processes mediated by *erα* and/or *erβ*[222,456]. In this study, the expression of *erα* mRNA were downregulated significantly excepting MP treatment group, while the expression of *erβ* mRNA was generally increased, with significant upregulated only in MA group. In zebrafish, the interplay and cross-regulation between ERα and ERβ is intricate, and each receptor possesses their distinct physiological functions[457]. This complexity might be an explanation for the observed divergent trends in the mRNA expression levels of these two receptors[452]. Their interaction in fish need additional investigations.

In all hormones level, there had significantly differences in BPA and BPS group suggesting that the impact of BPA on hormone levels in zebrafish is more sensitive than the effect of BPS. In the present study, we also could conclude that BPs and their co-exposure with MP altered the concentration of sex hormones and the expression of related genes, eventually influencing the reproduction of zebrafish[458]. It is well known that MP have the ability to adsorb BPs[459], which could potentially influence the bioavailability and toxicity of BPs to aquatic organisms, leading to more pronounced toxic effects compared to individual exposures. In this study, the co-presence of BPs

and MP may have enhanced the toxic effects observed in zebrafish, which is consistent with the results of other studies[33], potentially due to increased BPs adsorption on the MP particles. Notably, in our study, the co-exposure of MP with BPs has a more significant impact on hormone levels and related gene expression in zebrafish than single exposure to either substance, suggesting that the adsorption of BPs onto MP might exacerbate their combined toxicity on zebrafish reproductive systems. This highlights the importance of considering the interaction between MP and chemical pollutants in assessing environmental risk.

4.3 The common effect of parental exposure to BPA/S and MP alone and their co-exposure on zebrafish F1 generation by transcriptomic analysis

ABCC and ABCG are the subfamilies of ATP binding cassette (ABC) family, which are present in fish intestine[460]. In studies on fish, the ABCC subfamily transporters have been found to possess protective functions against toxic substances[461,462]. Furthermore, *abcg2* recognized as an essential gene for the detoxification of xenobiotics[463,464]. These reports and our present results together suggested that environmental pollutants such as BPA, BPS, and MP could impact the expression and function of genes in the ABC transporter pathways in fish. Most importantly, when fish are exposed to environments contaminated with toxic pollutants, the primary function of ABC transporters is to pump potentially hazardous out of the cells[460].

It is believed that apoptosis in fish could cause abnormal development during early-life stages[465]. Some researchers have indicated BPA and its substitutes could induce early apoptosis[253,466] and MP exposure significantly increased apoptosis level[117,467]. *Fosaa* is a member of the AP-1 (activating protein-1) family, is a crucial factor that regulate cell proliferation and apoptosis[468,469]. In this study, the expression levels of *fosaa* and *si:dkey-269i1.3* which are related to apoptosis were significantly upregulated in all treatment groups. Apoptosis is a process of cell death in response to the physiological and pathological signals, responding to the alterations in environmental conditions[470].

Furthermore, BPA, BPS and their co-exposure with MP together caused changes in the expression of *mcoln3a*, *LOC569566* and *camk1ga*, which are related to calcium signaling pathway. Calcium signaling plays a crucial role in the regulation of embryogenesis and mediating cell death through necroptosis[471,472]. Some investigations of the BPA and its alternatives have revealed they could interrupt calcium-mediated signaling[473] and cause excessive calcium influx and abnormal calcium signaling, along with inducing apoptosis[474]. The results of the present study demonstrated that parental exposure to BPA/S and MP alone and their co-exposure could together influence the

early stages of zebrafish offspring development through the apoptosis and calcium signaling pathways.

4.4 Differential effects of parental exposure to BPA and MP alone and their co-exposure on zebrafish F1 generation by transcriptomic analysis

Our results implied that BPA/S and MP may produce similar developmental toxicities to zebrafish offspring, and their combined effect have certain specificity. The neuroactive ligand-receptor interaction signaling pathway was directly associated with neurological function[475]. The *adra2c* gene has been shown to be associated with schizophrenia[476]. *Chrm2a* and *chrm5a*, are G protein-coupled receptor, which are extensively expressed in dopaminergic areas of brain[477,478]. Some reports showed that *chrm2* and *chrm5* were involved in neurological symptoms, abnormal synaptic dopamine release[479]. *Drd1b* and *drd2l* have the ability to bind dopamine, which belongs the family of dopaminergic receptor[335,480]. A recent study showed that dopaminergic receptors are associated with disturbances or malformations of the extracellular matrix of the nerve[481]. Gamma-aminobutyric acid type A receptor (*gabra6a*, *gabrb3*, *gabrd*, *gabrg2*, *gabrr2b* and *gabrz*), the primary inhibitory neurotransmitter in the central nervous system and serves as a powerful regulator of developmental and mature stages in neural networks[482,483]. The effect of *grin2a* and *grin2b* might be observed in neurodevelopment disorders[484]. *Npffr1* is widely distributed in the central nervous system, which is implicated in regulating feeding behavior in vertebrates[485]. In addition, expression levels of *npy8ar* and *ntsr1* which are encoding for neuropeptide receptor, are vital indicators for neurotoxicity and neuronal networks, respectively[486,487]. In general, the opioid receptors (*oprd1a* and *oprk1*) showed a wide pattern of distribution in the zebrafish nervous system plays a critical role in pain and addiction systems[488]. In summary, these data indicated that the signaling pathways associated with neuroactive ligand-receptor interaction are indeed activated by the co-exposure of BPA and MP in zebrafish offspring. The results also revealed that the neurotoxicity of BPA was significantly produced by the co-presence of MP, which may be caused by interactions between BPA and MP.

Voltage-dependent calcium channels (VDCCs) as primary mediator of neuromuscular transmission is implicated in impaired neuromuscular transmission in zebrafish[405]. In our results, all genes related to VDCCs (*cacna1ab, cacna1ba, cacna1bb, cacna1db, cacna1fb, cacna1ha, cacna1i, cacna2d2a, cacna2d4b, cacnb3b, cacnb4a*) were significantly upregulated in co-exposure MA group, leading to rapid elevation of intracellular Ca^{2+} levels and triggered various cellular responses, and eventually lead to pathological consequences[489]. Changes in the expression of any VDCCs are linked to

neurological disorders[490]. *Arrb1* has been observed in the pathophysiology of major depressive disorder[491,492] and *prkcg* gene could also play an important role in the development of major depressive disorder[493]. The ErbB receptors activate central signaling pathways which could regulate the organogenesis of vertebrates[494]. *Erbb4* could modulated neuromuscular development in zebrafish embryos[495]. Fu et al. (2023) found overexpression of *rps6ka2* could inhibit cell proliferation via MAPK signaling pathway[496]. All these findings suggested that co-exposure of BPA and MP could influenced neurotoxicity and neurological disorders via activating the MAPK signaling pathway and voltage-dependent calcium channels activity.

4.5 Differential effects of parental exposure to BPS and MP alone and their co-exposure on zebrafish F1 generation by transcriptomic analysis

The retina-specific members of the G protein-coupled receptor kinase (GRK) family, *grk1b* and *grk7a*, are involved in the phosphorylation process of rhodopsin and opsins. *Guca1b* (also known as *gcap2*) regulates the Ca^{2+} sensitive activation of retinal guanylate cyclases. These three genes are essential for participating the recovery of vertebrate visual phototransduction[497,498]. *Gucy2f* is a membrane-bound guanylate cyclase[499] and its elimination could markedly reduce visual response[500]. *Rcvrna*, *rcvrn2* and *rcvrn3* are recoverin genes in zebrafish retina, which could modulate the cone phototransduction cascade[501]. *Rgs9a* might be associated with the light-sensing ability of the eye, and alterations in its expression could influence the sensitivity of light response recovery[502].

Retinoic acid are derivatives of retinol (vitamin A) which are crucial for numerous biological processes, including visual function, growth, development and reproduction[503,504]. There has been extensively examined that the cytochrome P450 enzyme system is involved in the oxidative metabolism of retinoic acid[504,505]. Retinoic acid can also be degraded into metabolites with greater polarity and water solubility by UDP-glucuronosyltransferase (ugt)[506]. In this study, *cyp3c3* gene of cytochrome P450 enzyme family and *ugt1a2*, *ugt1a5*, *ugt1b2*, *ugt2a1*, *ugt2a2* and *ugt2a6* of UDP-glucuronosyltransferase family were crucial unique differential genes in MS group to regulate the retinol metabolism pathway. The results also revealed that the unique differential genes in MS group may exert adverse impacts on visual development and toxicity through phototransduction and retinol metabolism, compared to that exposed to BPS and MP alone.

4.6 The common effect of parental exposure to BPA/S and MP alone and their co-exposure on zebrafish F1 generation by metabolomics analysis

Among the 17 shared differential metabolites in the five treatment groups, 8 were enriched in glycerophospholipid metabolism. Most of these glycerophospholipid metabolites were upregulated in all treatments (PC(16:0/0:0), PC(18:1(9Z)/0:0), PC(20:4(8Z,11Z,14Z,17Z)/0:0, PC(20:3(8Z,11Z,14Z)/0:0), PE(20:1(11Z)/22:5(7Z,10Z,13Z,16Z,19Z), PC(18:1(11Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)), and two of them were downregulated (PC(16:1(9Z)/20:2(11Z,14Z)), PC(16:0/18:2(9Z,12Z))). Glycerophospholipids, which are primary components of cellular membranes and precursors to lipid mediators, are closely associated with numerous metabolic diseases, including neurological diseases, cardiovascular disease, fatty liver and cancer[507,508]. Besides, it is reported that BPs[509,510] and MP fibers[511] could induced dysregulation in glycerophospholipids which are involved in cell membrane damage and oxidative damage. Then, the alterations of glycerophospholipids after five different treatments indicated that BPA/S and MP exposure alone or their co-exposure could together influence the oxidative damage and disturbances of cellular homeostasis.

4.7 Differential effects of parental exposure to BPA or BPS and MP alone and their co-exposure on zebrafish F1 generation by metabolomics analysis

Organ damage caused by environmental pollutants could impact the metabolism of organisms, including lipid metabolism and carbohydrate metabolism[512]. Arachidonic acid (ARA) is an essential PUFA (polyunsaturated fatty acid), which could regulate inflammation, blood pressure, growth and reproduction in zebrafish[513]. The significant enrichment in arachidonic acid metabolism demonstrated that co-exposure of BPA and MP inhibited the phospholipid metabolism capability in F1 zebrafish embryos. In this study, based on the elevated metabolites, such as pge2, 15-F2t-Isop (also known as 8-isoprostane) and 12-Oxo-Ete, played an important role in immune system function and antioxidative activities[514]. The results suggested that zebrafish embryos did suffer from oxidative stress and immune system dysregulation after co-exposure of BPA and MP, compared to their exposure alone.

Furthermore, the metabolomics results indicated dysregulation of pyruvate metabolism and glycolysis/gluconeogenesis metabolism upon the co-exposure of BPS and MP, which were belong to carbohydrate metabolism. Carbohydrate metabolism is a critical

biological process in zebrafish metabolism, which significantly influence the growth and development[515,516]. It is worth mentioning that the co-exposure of BPS and MP induced unique differential genes (such as: *adpgk2* and *gck*) and unique differential metabolites (such as: L-Lactate) are involved in the glycolysis/gluconeogenesis metabolism (Figure 5-5D and 5-8D), indicating co-exposure of BPS and MP activated the glucose metabolism. Taken together, these data indicated that parental exposure to BPA, BPS, MP and their co-exposure may disrupt different basic cellular mechanisms and different developmental processes.

Zebrafish, serve as a well-established model, is being increasingly utilized for toxicological studies due to their genetic and physiological similarities to human[517,518]. Zebrafish share key molecular pathways with humans, particularly in reproductive and endocrine functions[242]. The results of this study could provide important clues about potential health risks in mammal animals and human, especially concerning reproductive toxicity and transgenerational effects. Therefore, further research and epidemiological studies will be essential to fully understand the implications of these findings for mammal animal and human health.

5. Conclusions

Our results demonstrated that the parental exposure of BPs and their co-exposure with MP exhibited different degrees of effects on reproductive toxicity to adult zebrafish through the alterations of hormones concentration and genes in the HPGL axis. Combined toxicity of BPs with MP had more significant impacts on reproductive toxicity than single exposure. Transcriptomics and metabolomics further showed combined exposure of BPs and MP could existed similar toxicities to F1 generation of zebrafish, and the effect of their co-exposure also have unique differences. Overall, this study investigated single or co-exposure of BPs and MP induced negative effects in adult fish and improved understanding of the underlying mechanisms of single or co-exposure of BPs and MP's toxicity on zebrafish offspring. The combined toxicity risk assessment should be considered not only consider the single exposure. In addition, this study highlights the necessary of more research on the transgenerational toxic from exposed parents to their offspring.

6. Supplementary data

Table S5-1. Sequences of primers for the target genes related to HPGL axis

Gene	Forward primer	Reverse primer
gnrh2	GGTCTCACGGCTGGTATCCT	TGCCTCGCAGAGCTTCACT
gnrh3	TGGTCCAGTTGTTGCTGTTAGTT	CCTGAATGTTGCCTCCATTTC

gnrhr2	ACAGCGTGAGCAAAACATTG	TGAGCACAAACTCAGCATCC
gnrhr3	AACAGACATGATCCCGAAGG	AGGTTCCCGAACACAAACAG
lhβ	GGCTGGAAATGGTGTCTTCTT	GGAAAACGGGCTCTTGTAAAC
lhr	CCTGGTCGTCCTGCTGGTT	AAGGCTAGATGGCACATTAGAAATC
fshβ	GCAGGACTATGCTGGACAATG	CCACGGGGTACACGAAGACT
fshr	CGTCTCTTTTGTGCACTGGA	GTGGCAATTCCACACTTCCT
hmgr	TCAACTGGATTGAGGGAAGG	AGCGTTATAACCACCGATGC
star	CCTGGCATTGGAAAGGTTTTT	GTTCCATGTTATCTACCAGCTCATCA
cyp11a	ACAGCCTGCTCAGTGTCCTT	AGCACCGTCTTCAGGCTTTA
cyp19a	GCTGACGGATGCTCAAGGA	AAACGTCCACCACGATGCA
cyp19b	AACCTCTCCATGCAGCCAGTAG	TGAGCTCGCGGGATGAAG
erα	GGTCCAGTGTGGTGTCCTCT	CACACGACCAGACTCCGTAA
erβ	AGCTTGTGCACATGATCAGC	GCTTTCA TCCCTGCTGAGAC
ar	ACATTCTGGAGGCCATTGAG	ACGTGCAAGTTACGGAAACC
vtg1	AACGAACAGCGAGAAAGAGATTG	GATGGGAACAGCGACAGGA
β-actin	CGAGCAGGAGATGGGAACC	CAACGGAAACGCTCATTGC

Table S5-2. Sequences of primers for DEGs from RNA-seq and RT-PCR in offspring

Gene	Forward primer	Reverse primer
abcc8b	CCTGAGAACTATGAAGGACTGC	ACTTCCCACTTCCTGTCCTG
clul1	CTTTGGCAGACGTGTGTTGG	GACTCCCTGGCAGACTTCAC
fosaa	CCTCCGTGTCTCCGTCTCAA	CTGCTCGCTCTTGCTCTTCC
hbbe3	ATGGTTGTGTGGACAGCTG	ATTATCCATGTTGTTGAGAGC
mfn1a	ACAGGTCGACAAGAGCGAGA	CAGATCAGTGGCTTTGTGTCTGA
opn1sw1	CGAGAGATATGTGGTCATCTG	TGTATCTGCTCCATCCAAAG
pdha1b	TGATTAGCAGCAACATGGCCA	GCCTCTGACCTCTAATGGAGCA
prkar2ab	GATCATACGGCAGGGCGATT	GTTTGACCTCTGACCTGCGT
prph2b	GGCTTGCGGTGTCAATACTT	CTGGTGTGTCGGTGTCTTTG
β-actin	CGAGCAGGAGATGGGAACC	CAACGGAAACGCTCATTGC

Table S5-3. Instrumental operating parameters of UHPLC-QE Orbitrap MS for metabolomics analysis

	Parameter		
Instrument	Thermo Scientific ultrahigh performance liquid chromatography system (UHPLC) coupled to a Q Exactive Focus Hybrid Quadrupole-Orbitrap Mass Spectrometer (QE Orbitrap MS)		
Column	Waters HSS T3 (100 mm× 2.1 mm, 1.8μm)		
Column temperature	45°C		
Mobile phases	A: 0.1% formic acid in H_2O; B: acetonitrile		
Gradient profile	Time (min)	B%	Flow rate (mL/min)
	0.0	5	0.35
	2.0	5	0.35
	4.0	30	0.35
	8.0	50	0.35
	10.0	80	0.35
	14.0	100	0.35
	15.0	100	0.35
	15.1	5	0.35
	16	5	0.35
Injection volume	3μL		
MS Parameters	Acquisition mode: Data dependent acquisition (DDA)		
	Spray voltage: 3800V (positive ionization mode), -3000V (negative ionization mode)		
	Capillary temperature: 320°C		
	Auxiliary gas heater temperature: 350°C		
	Sheath gas flow rate (arbitrary units): 35		
	Auxiliary gas flow rate (arbitrary units): 8		
	S-lens RF level: 50		
	Mass range (*m/z*): 100–1200		

Full MS resolution: 60000 MS/MS resolution: 15000 Collision energy: 10 eV, 20 eV, 40 eV.

Text S5-1. Detailed RNA sequencing and differentially expressed genes analysis

Thirty milligrams of embryos samples were precisely weighed and dispensed into a 1.5mL Eppendorf tube (n=3). The sequencing libraries were processed on an Illumina Novaseq 6000 platform and 150bp paired end reads. Initially, these raw reads, in FASTQ format, were subjected to preprocessing with fastp, where reads of inferior quality were excised, resulting in a collection of high-quality, or "clean," reads. Subsequently, each sample were preserved for further analysis. These clean reads were aligned to the zebrafish genome (ID: Danio rerio GRCz11) utilizing the HISAT2 tool. The expression levels of genes, quantified as Fragments Per Kilobase of transcript per Million mapped reads (FPKM), alongside the read counts for each gene, were derived using HTSeq-count. Principal Component Analysis (PCA) was executed utilizing R software (version 3.2.0) to assess the sample biological replicability.

Differential expression analysis was performed using the DESeq2. Q value < 0.05 and foldchange > 2 or foldchange < 0.5 was set as the threshold for significantly differential expression gene (DEGs). Hierarchical cluster analysis of DEGs was performed using R (v 3.2.0) to demonstrate the expression pattern of genes in different groups and samples. The radar map of top 30 genes was drawn to show the expression of up-regulated or down-regulated DEGs using R packet gradar.

Based on the hypergeometric distribution, GO (Gene Ontology), KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway, Reactome and WikiPathways enrichment analysis of DEGs were performed to screen the significant enriched term using R (v 3.2.0), respectively. R (v 3.2.0) was used to draw the column diagram, the chord diagram and bubble diagram of the significant enrichment term.

Gene Set Enrichment Analysis (GSEA) was performed using GSEA software. The analysis was used a predefined gene set, and the genes were ranked according to the degree of differential expression in the two types of samples. Then it is tested whether the predefined gene set was enriched at the top or bottom of the ranking list.

Text S5-2. The detailed process of metabolites extraction

Thirty milligrams of embryos samples were precisely weighed and dispensed into a 1.5mL Eppendorf tube before adding two diminutive stainless-steel beads (n=6). Subsequently, 400μL of a methanol-water solution (volumetric ratio 4:1), containing a mixed internal standard at a concentration of 4μg/mL, was introduced into the tube. Prepared samples were subjected to a pre-chilling process at -40°C for a duration of 2 minutes, followed by homogenization using a bead mill operating at a frequency of 60 Hz for 2 minutes. Post-milling, samples were exposed to ultrasonic extraction in an ice-water bath for 10 minutes, after which they were allowed to stand at -40°C overnight to ensure thorough extraction. Centrifugation was subsequently performed for 10 minutes at 12000 rpm and 4°C, after which 300μL of the resulting supernatant was carefully transferred into an LC-MS sample vial and the solvent was evaporated until dry. For reconstitution, 300μL of methanol-water (volumetric ratio 1:4) was added to the dried residue. This was followed by vortex mixing for 30 seconds and sonication in an ice-water bath for 3 minutes. Samples were then left to stand at -40°C for 2 hours to facilitate complete reconstitution. A second centrifugation step was performed under identical conditions as before, and 150μL of the clear supernatant was extracted using a syringe. This extract was then passed through a 0.22μm organic phase syringe filter to remove any particulate matter and stored at -80°C until further analysis. All extraction solvents were pre-cooled to -20°C prior to their use in the protocol, ensuring the maintenance of optimal conditions for sample preparation and analysis. For the purposes of quality control, Quality Control (QC) samples were meticulously prepared by combining equal volumes of extracts from all the processed specimens.

Text S5-3. Detailed data analysis for metabolomics

For the raw metabolomics data, we used MS-DIAL (Version 4.38) software for the peak deconvolution. Subsequently, fragment ions that had less than 20% missing values were selected, after which these ions exhibiting a relative standard deviation greater than 20% were eliminated, using sum normalization as the filtering criterion.

In addition, principal components analysis (PCA) and projection to latent structure-discriminant analysis (PLS-DA) were employed using log-normalized, mean-centered, and variance-scaled data.

Metabolites that showed significant changes between the control group and the exposure group were identified by selecting those with a Variable Importance in Projection (VIP) score greater than 1 from Partial Least Squares Discriminant Analysis (PLS-DA), alongside a p-value of less than 0.05. This p-value was determined through an independent t-test comparing metabolite levels in the exposure group with those in the control group.

Finally, the metID software and Human Metabolome Database (HMDB, http://www.hmdb.ca/) were used to structurally identify the differential metabolites with MS/MS fragments. Metabolic pathways analysis was carried out by Danio rerio Kyoto Encyclopedia of Genes and Genomes (KEGG) library (https://www.kegg.jp/kegg/pathway.html).

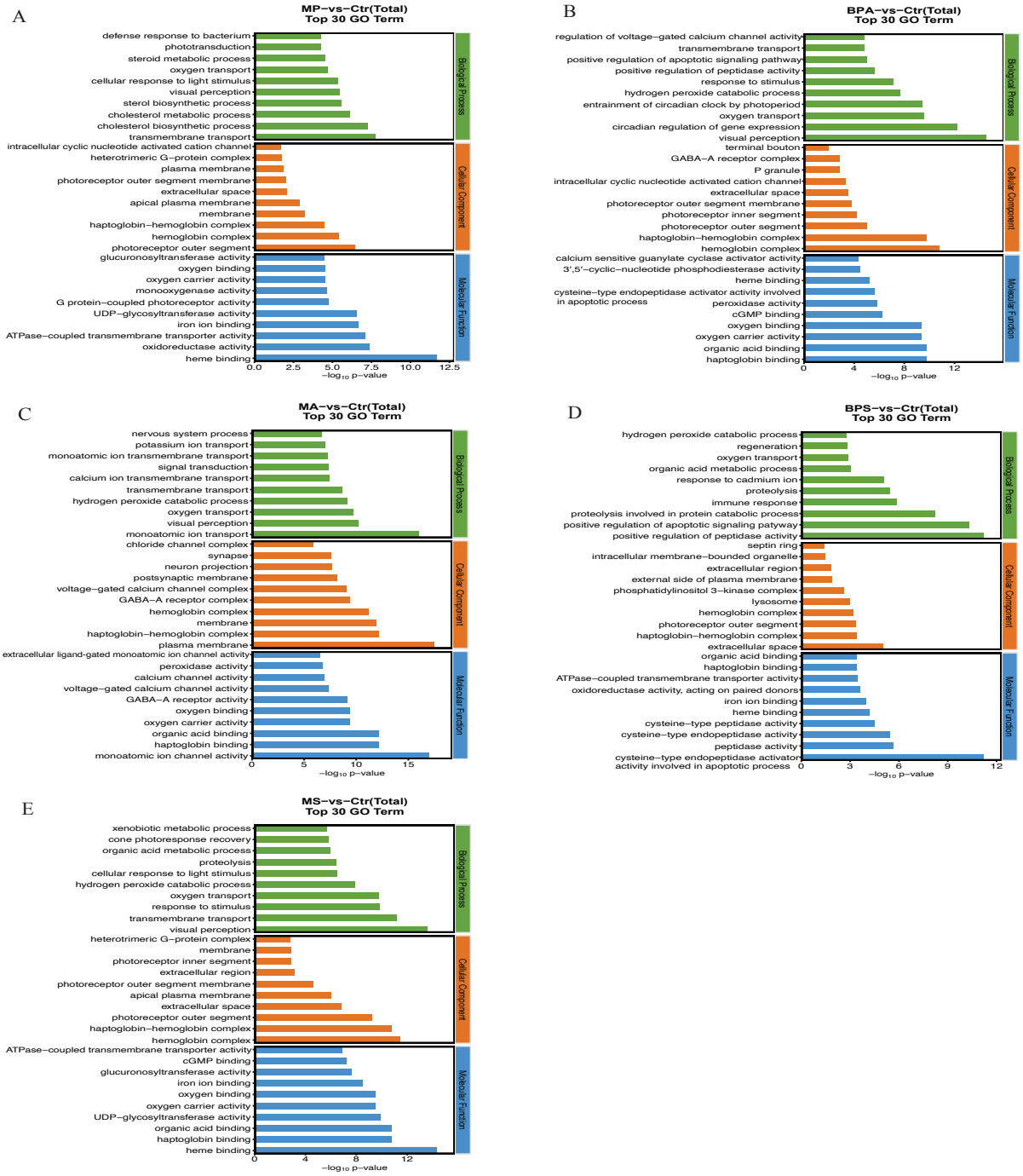

Figure S5-1. The enriched GO terms of DEGs for biological process, cellular component and molecular. A: MP group compared with the control group. B: BPA group compared with the control group. C: MA group compared with the control group. D: BPS group compared with the control group. E: MS group compared with the control group.

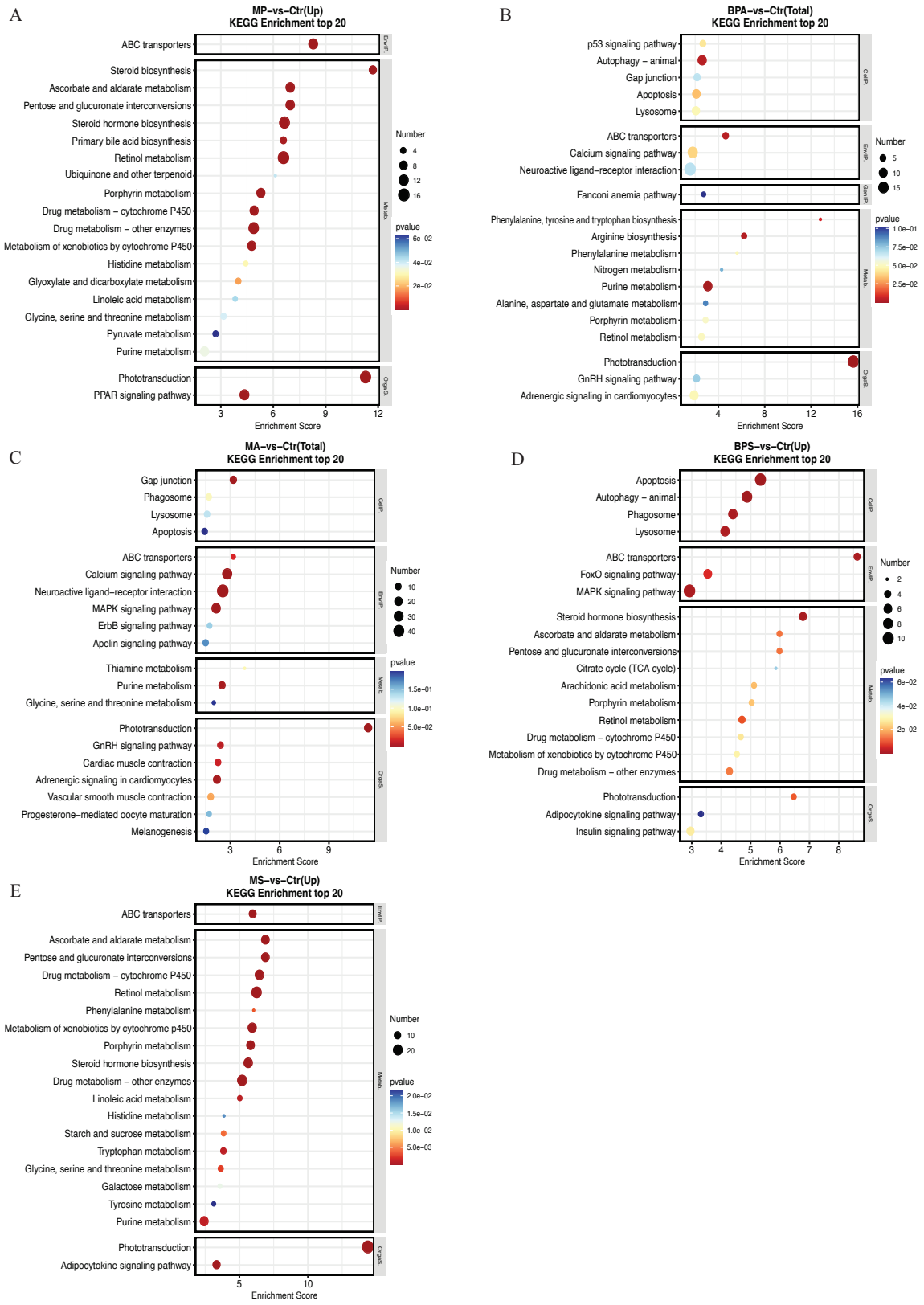

Figure S5-2. KEGG enrichment scatter diagram. A: MP group compared with the control group. B: BPA group compared with the control group. C: MA group compared with the control group. D: BPS group compared with the control group. E: MS group compared with the control group.

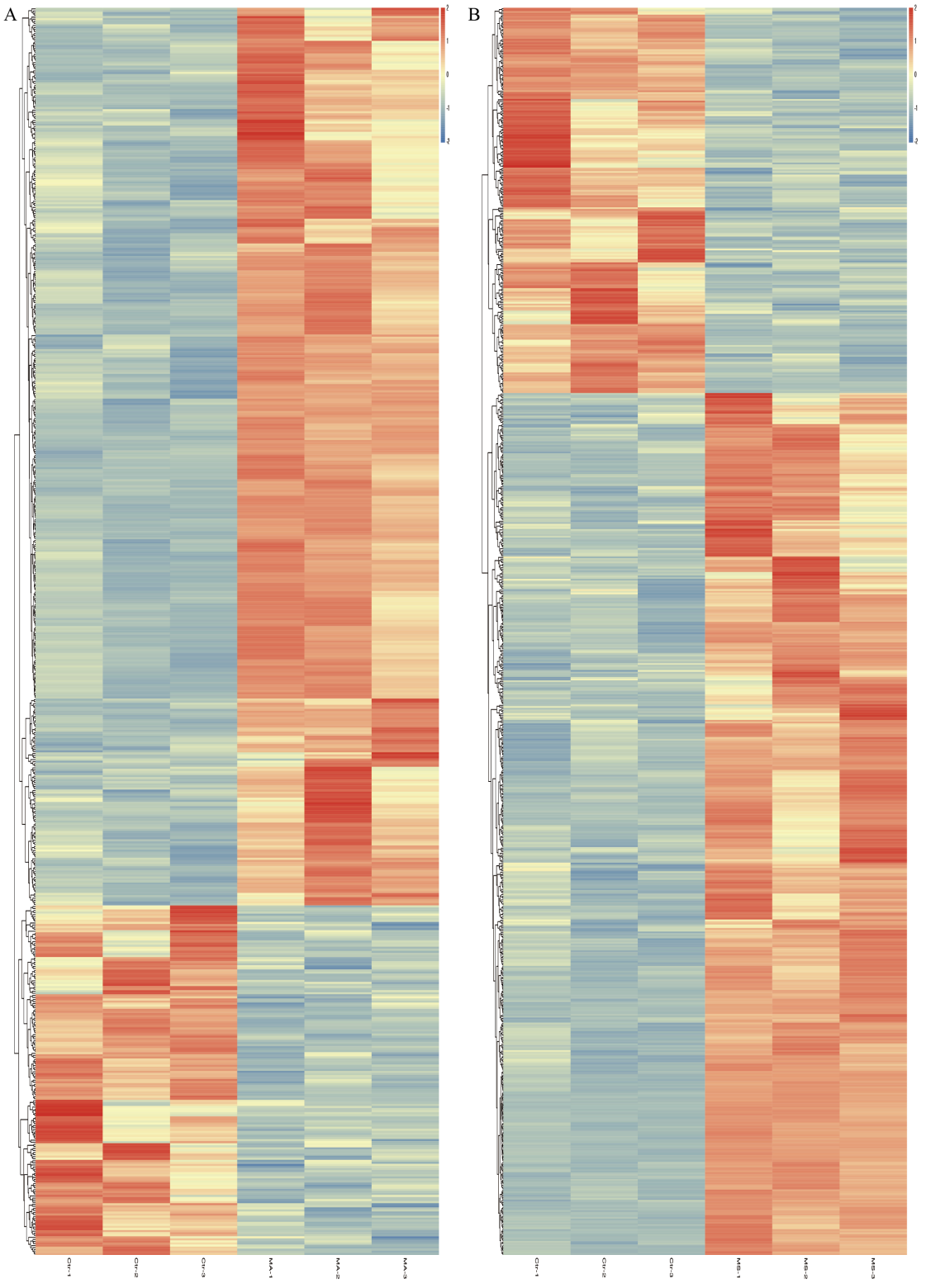

Figure S5-3. Heatmaps of these unique differential genes in co-exposure groups of MA group (A) and MS group (B).

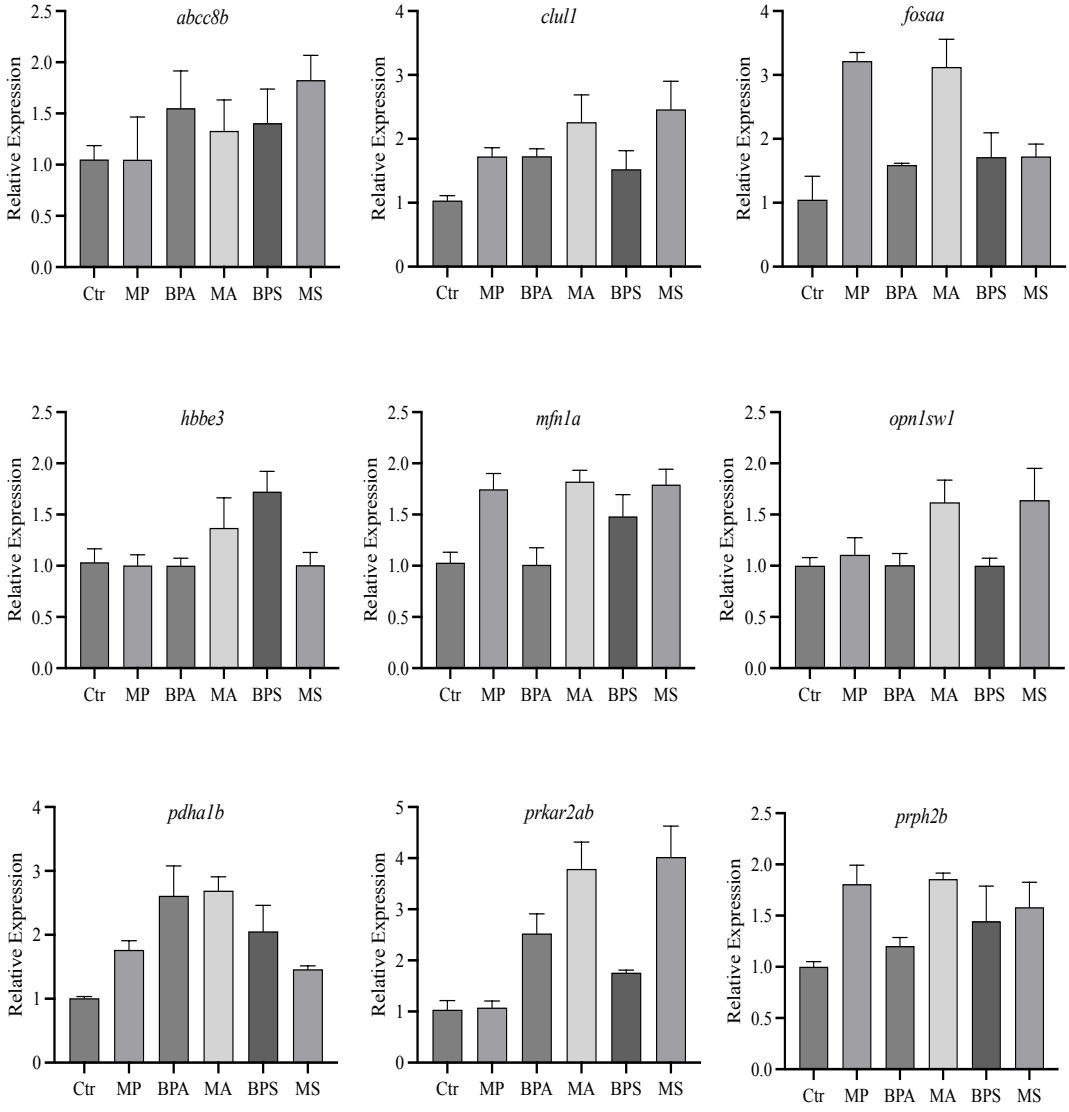

Figure S5-4. Relative transcript levels of DEGs from RNA-seq and RT-PCR in offspring.

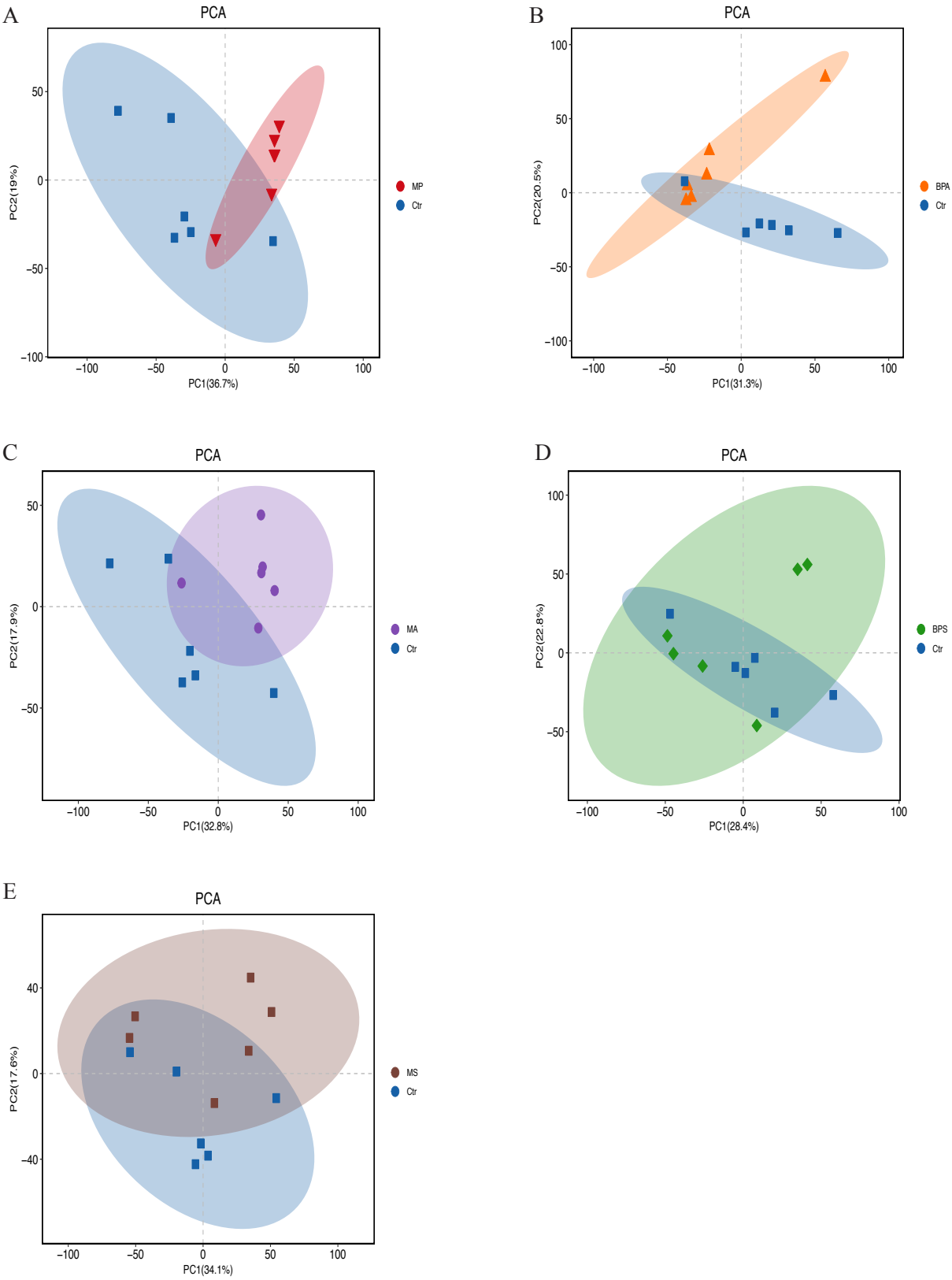

Figure S5-5. Score plots of PCA in metabolomics of zebrafish embryos. A: MP group compared with the control group. B: BPA group compared with the control group. C: MA group compared with the control group. D: BPS group compared with the control group. E: MS group compared with the control group.

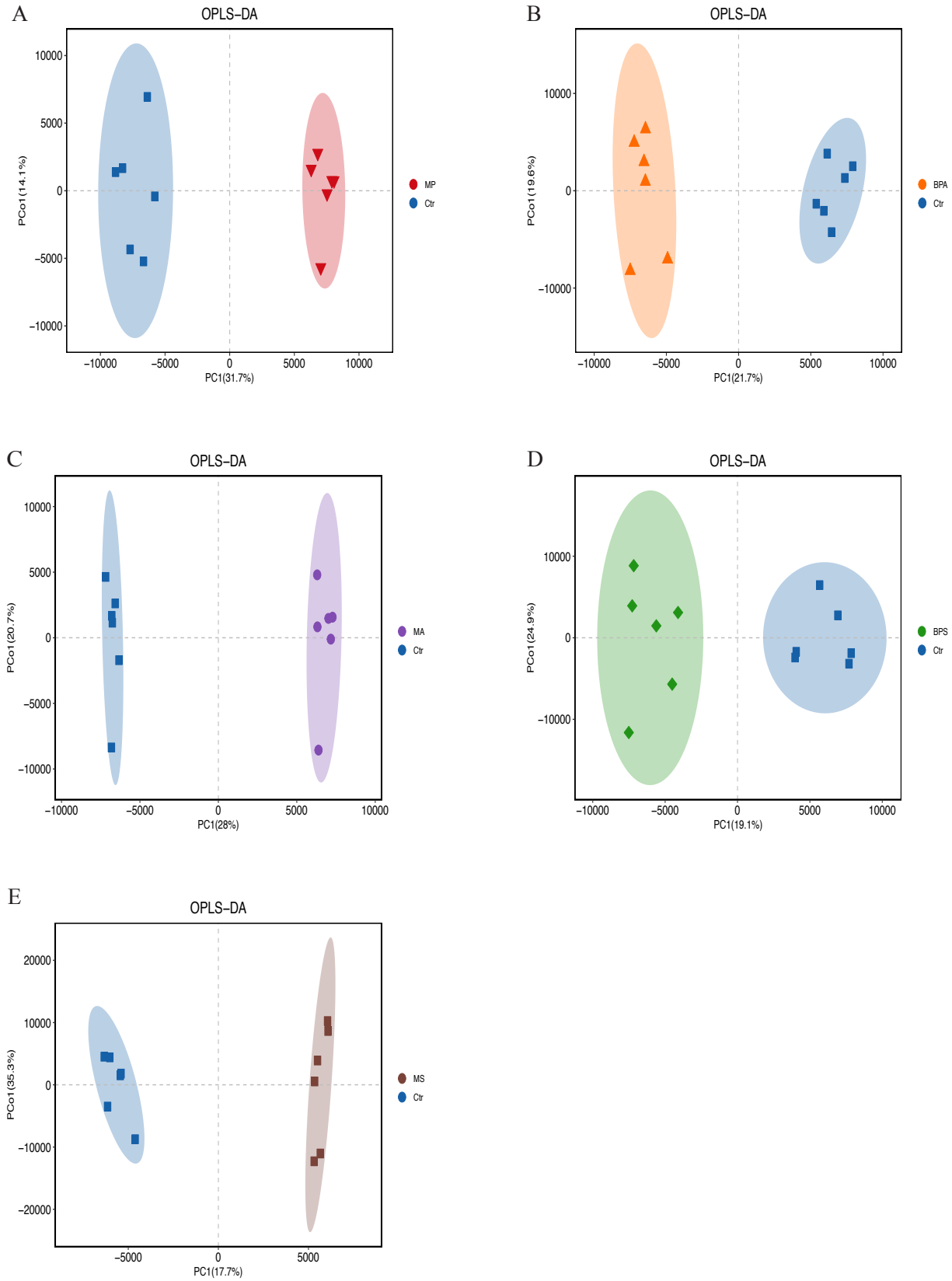

Figure S5-6. Score plots of OPLS-DA in metabolomics of zebrafish embryos. A: MP group compared with the control group. B: BPA group compared with the control group. C: MA group compared with the control group. D: BPS group compared with the control group. E: MS group compared with the control group.

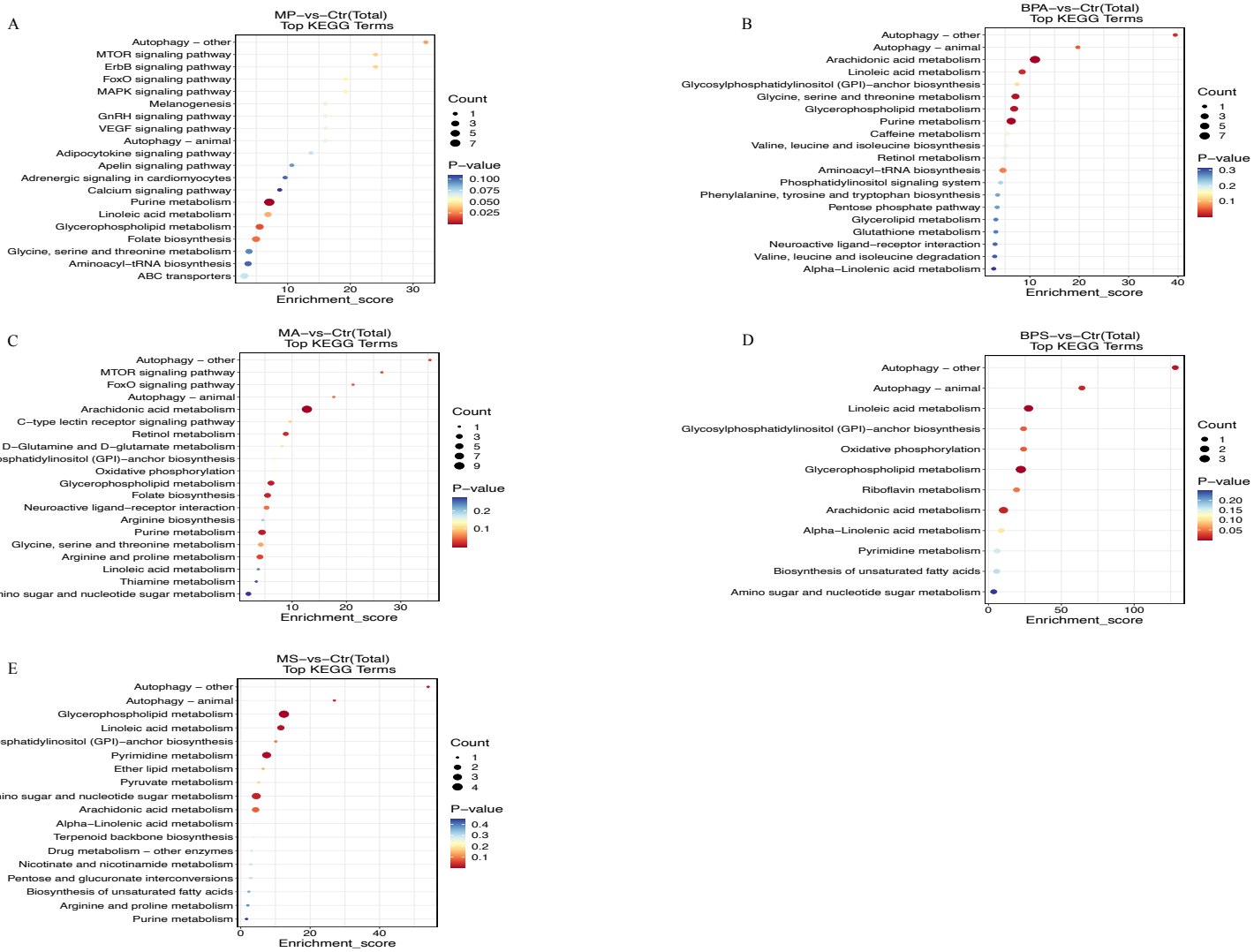

Figure S5-7. The enriched KEGG pathways of differential metabolites of zebrafish embryos. A: MP group compared with the control group. B: BPA group compared with the control group. C: MA group compared with the control group. D: BPS group compared with the control group. E: MS group compared with the control group.

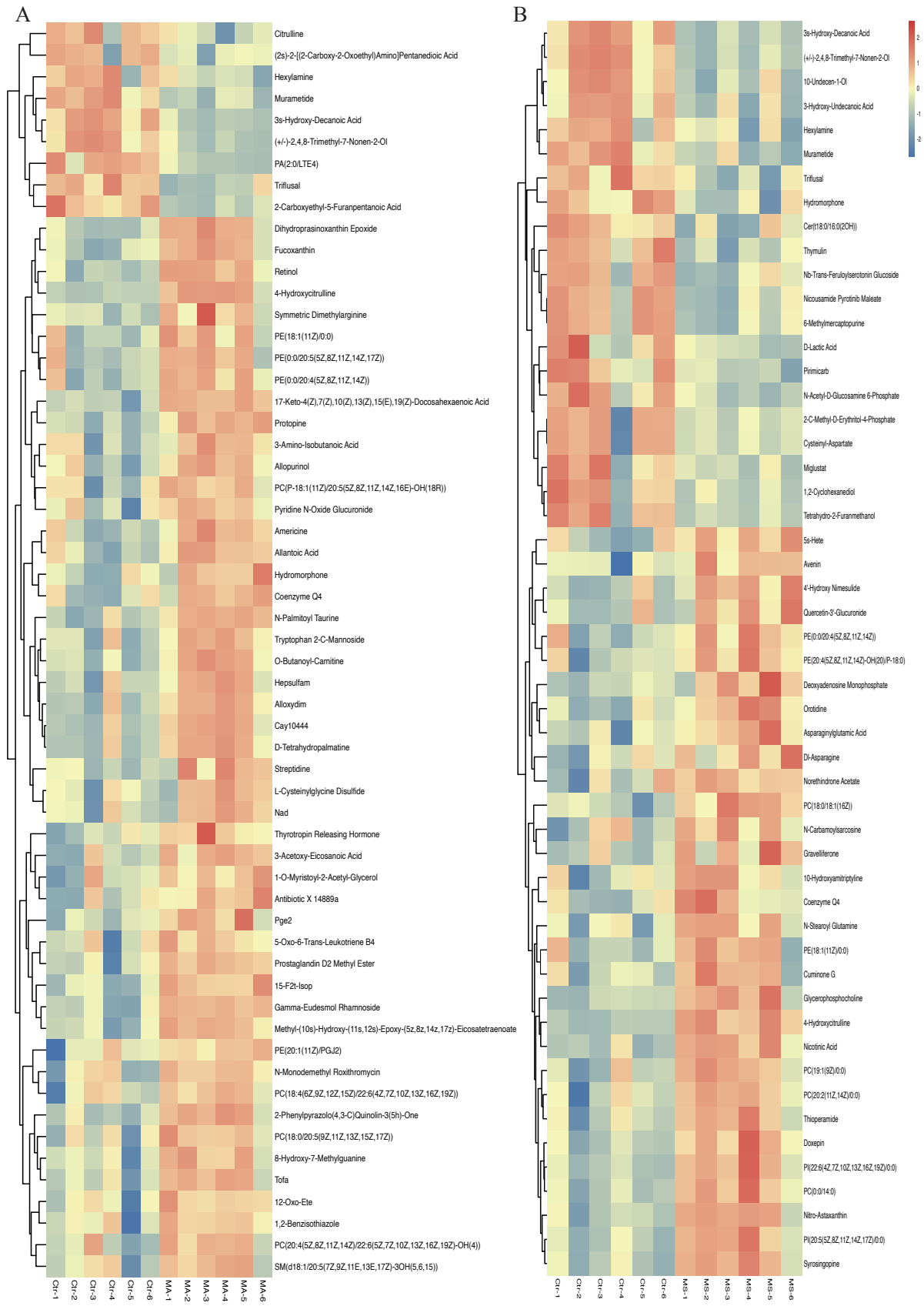

Figure S5-8. Metabolomics heatmap of unique differential metabolites in MA group (A) and MS group (B).

Chapter 6

General discussion, conclusions and perspectives

1. General discussion

Microplastics (MP), an emerging pollutant, which is prevalent in all kinds of environments[519]. MP could harm aquatic organisms by causing oxidative stress, immune disruption, and reproductive issues[132], while bisphenol compounds (BPs) act as endocrine disruptors, altering hormonal balance and reproductive functions[520]. The coexistence of MP and BPs in aquatic environments presents a compounded threat, as MP facilitate the transportation and magnification of BPs' harmful effects. Studies have shown that co-exposure to MP and BPs exacerbates adverse effects such as reproductive failure and neurotoxicity, significantly more than single exposures to either pollutant[521,522]. This underlines the necessity of studying these pollutants together, as their combined impact poses long-term ecological risks that extend beyond individual organisms to affect entire populations and ecosystems.

Research into the combined effects of MP and BPs is essential for understanding their full environmental and biological impacts. As these pollutants continue to accumulate, there is a pressing need for strategies that address their combined presence in ecosystems to mitigate the risks they pose to aquatic life and, potentially, human health through the food chain[7,8,10,11]. Furthermore, beyond the measurements conducted in this study, it is crucial to consider the broader implications of these findings in real-life environmental and human health contexts.

1.1 Bioaccumulation and toxicological risks of bisphenol compounds and microplastics in zebrafish

Our results demonstrated a sensitive and accurate UPLC-MS/MS method to quantify bisphenol A (BPA) and bisphenol S (BPS) in zebrafish tissues and examined the bioaccumulation of these compounds with and without the presence of polyethylene microplastics (MP). The results clearly demonstrated that both BPA and BPS accumulate significantly in zebrafish tissues, particularly in detoxification organs such as the liver and intestines. Moreover, the co-exposure to MP substantially increased the bioavailability of BPs, leading to higher concentrations in zebrafish tissues compared to exposure to BPs alone. This enhancement in bioaccumulation due to MP reinforces the "Trojan horse" hypothesis[311,322], wherein MP serve as vectors, increasing the accessibility of hydrophobic contaminants to organisms[523]. This method allowed for precise quantification of BPs accumulation across different tissue types, providing critical insights into the tissue-specific distribution of these compounds. The significant accumulation in the liver and intestines reflects the body's attempt to metabolize and detoxify these pollutants, further indicating that co-exposure to MP accelerates bisphenol uptake and storage[524].

Much of the earlier research on BPs and MP have focused on their individual effects, this study highlights the importance of investigating co-exposure conditions, which are more representative of real environmental conditions. The enhanced bioavailability and tissue-specific accumulation due to the presence of MP underline the critical need for revised risk assessments that consider the cumulative effects of pollutant mixtures, rather than single substances[33].

This study lays a critical foundation for the further toxicological investigations. The enhanced bioaccumulation of BPs in the presence of MP provides a mechanistic basis for the oxidative damage of zebrafish observed in the following chapters. The data on how MP increase BPs concentrations in specific tissues, such as the liver and intestines, explain the heightened oxidative stress and cellular damage mechanisms in the thesis. Additionally, the bioaccumulation patterns established are directly relevant to the transgenerational effects, where the impact of parental exposure on offspring development and survival is linked to the pollutants' increased presence in biological systems.

Thus, this part of the study not only provides critical data on the bioavailability of BPs after co-exposure with MP in zebrafish but also sets the stage for understanding how these pollutants exert their toxic effects at both cellular and generational levels. The insights on pollutant bioaccumulation are vital for advancing the field of ecotoxicology, providing the methodological and theoretical groundwork for subsequent studies on the combined effects of MP and chemical pollutants.

However, a key limitation of this study is the controlled laboratory setting, which does not fully replicate real-world environmental conditions. Natural ecosystems are complex and influenced by multiple factors such as water flow dynamics, microbial degradation, and interactions with other pollutants, all of which could influence the bioaccumulation and toxicity of bisphenol compounds. Further studies are needed to explore these interactions in more complex environmental models, including mesocosm or field-based studies.

1.2 Oxidative stress and cellular damage from co-exposure to microplastics and bisphenol compounds

Our results indicated that the oxidative damage caused by BPA, BPS, and MP, both individually and in combination in zebrafish. The study revealed that co-exposure to BPA/BPS and MP significantly increased oxidative stress compared to individual exposures. Markers of oxidative stress, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), were notably downregulated, indicating the impaired capacity of zebrafish tissues to neutralize reactive oxygen species (ROS). The liver and intestines were particularly affected due to their central roles in detoxification.

Additionally, apoptosis, as a downstream result of oxidative stress, was detected, further emphasizing the cellular damage induced by the combined exposure.

Our findings underscored the synergistic toxicity of MP and BPs in aquatic environments. Oxidative stress is a critical pathway through which environmental pollutants exert their toxic effects. The results revealed that the presence of MP exacerbates the oxidative stress induced by BPs, which aligns with the concept that MP can enhance the bioavailability of hydrophobic pollutants and significantly impairs the antioxidant defense mechanisms of fish[525,526]. The downregulation of antioxidant enzymes such as SOD, CAT, and GPx is a significant finding, as these enzymes are central to maintaining cellular redox balance[527]. Their suppression indicates a direct impairment of the fish's ability to detoxify the oxidative stress generated by the pollutants. This further highlights the compounded risk posed by co-exposure to MP and BPs, which aggravates the toxicity beyond what is seen with individual pollutants alone[528].

Moreover, the increased apoptosis observed in this study points to severe cellular damage that could have far-reaching consequences for the organism's overall health and reproductive capacity. Apoptosis not only impacts tissue function but could also lead to long-term disruptions in organ systems vital for metabolism and detoxification[273]. These results emphasize the need for further research on chronic exposures to better understand how these pollutants might affect the long-term viability of populations in natural ecosystems. This research is crucial for advancing the understanding of the molecular and cellular mechanisms through which combined environmental pollutants exert their toxic effects. While previous studies have established that both MP and BPs can individually cause oxidative stress[131,529,530], the co-exposure in this study provides new insights into how these pollutants interact to exacerbate biological damage.

These foundational results are crucial for understanding the mechanisms that contribute to the developmental and reproductive defects observed in the offspring of exposed zebrafish. The increased oxidative stress and cellular damage suggested that parental exposure to these pollutants could affect the health and viability of future generations. The link between oxidative stress in parents and developmental abnormalities in offspring underpins the rationale for investigating cross-generational toxicity, providing a more comprehensive view of the long-term ecological risks posed by MP and BPs[306,531]. In conclusion, this part provides critical insights into how the combined exposure to MP and BPs exacerbates oxidative stress and cellular damage. These findings not only deepen the understanding of the toxicological pathways involved but also set the stage for further investigation into the long-term, transgenerational impacts of these pollutants.

However, one of the limitations is that oxidative stress biomarkers were measured at specific time points, whereas in natural conditions, exposure and effects may be more

dynamic. Future studies should focus on real-time monitoring of oxidative stress responses using advanced techniques such as metabolomic profiling or biomarker-based biosensors in environmental monitoring programs.

1.3 Reproductive toxicity and transgenerational effects of bisphenol compounds and microplastics in zebrafish

This study explored the reproductive toxicity of BPA, BPS, and MP on adult zebrafish, as well as the transgenerational effects on their offspring. One of the major findings was the substantial reproductive toxicity in adult zebrafish after combined exposure to BPs and MP. Histological analysis showed that gonadal tissues in the co-exposure groups suffered more severe pathological damage than in single exposure groups. These effects are linked to the disruption of hormone levels, particularly estrogen (E2), testosterone (T), and 11-keto testosterone (11-KT), which were significantly reduced in co-exposure groups compared to control and single exposure groups. These structural changes in gonadal tissues align with disruptions in hormone balance. And the alteration in hormonal profiles is likely due to interference with the HPGL axis, which controls reproduction through a complex network of hormones and feedback loops[532,533]. The HPGL axis plays a crucial role in regulating reproductive functions in zebrafish[437], and this study demonstrated that combined exposure to bisphenols and MPs significantly affected the expression of key genes in this pathway. Previous studies have similarly reported that environmental endocrine-disrupting chemicals, such as BPs, can impair reproductive functions by altering the HPGL axis at both the genetic and hormonal levels[378,534]. These results suggest that MP enhance the bioavailability of BPs, intensifying their disruptive effects on hormonal regulation and gonadal development. The interaction between MPs and BPs amplifies the toxicity, leading to more profound reproductive damage than exposure to BPs alone. This supports previous studies indicating that MP can act as vectors, increasing the bioavailability and toxic impact of pollutants in aquatic environments[306,310].

Another key question was whether the reproductive toxicity observed in adult zebrafish could transmitted to their offspring. The study utilized transcriptomic and metabolomic analyses to investigate the impacts on the F1 generation. The results revealed significant transgenerational effects, indicating that the reproductive toxicity experienced by the parent zebrafish led to developmental abnormalities and reproductive dysfunction in their offspring. Additionally, metabolomic analysis showed that co-exposure groups had unique metabolic profiles compared to single exposure groups. Arachidonic acid metabolism was significantly affected in the F1 offspring of zebrafish exposed to BPA and MPs, indicating an inflammatory response and oxidative stress. These findings are consistent with previous studies that have demonstrated the heritable effects of endocrine disruptors like BPA, which can cause epigenetic changes that influence gene

expression across generations[535,536]. The present study extends this understanding by showing that the combined exposure to bisphenols and microplastics has even more pronounced transgenerational effects, disrupting critical developmental processes and metabolic pathways in zebrafish offspring.

This research highlights the long-term ecological and evolutionary risks posed by the combined exposure of BPs and MP. The study's findings emphasize that the reproductive and developmental effects of these pollutants are not limited to the exposed generation but can be passed on to next generation, potentially leading to population declines in polluted ecosystems. Given the ubiquity of MP and BPs in aquatic environments, understanding the transgenerational toxicity of these compounds is essential for assessing their long-term impacts on biodiversity and ecosystem health. Moreover, this study provides critical evidence that the interaction between MP and BPs enhances their toxic effects, which must be considered in environmental risk assessments.

Although this study provides strong evidence of transgenerational effects, it remains unclear whether these effects persist beyond the F1 generation. Future studies should explore F2 and F3 generations to determine whether the observed effects are reversible or accumulate over successive generations. Additionally, expanding research to other aquatic species would help assess whether these findings are applicable across different taxa.

2. Broader implications and real-life relevance

While this study focused on zebrafish as a model organism, its implications extend beyond aquatic ecosystems. Microplastics and bisphenol compounds are pervasive environmental contaminants, and their effects on human health and other wildlife warrant further investigation.

2.1 Implications for human exposure

Humans are continuously exposed to bisphenol compounds and microplastics through various pathways, including dietary intake (e.g., contaminated seafood, bottled water, and packaged food), inhalation, and dermal absorption. The results of this study raise important concerns regarding potential health risks, particularly in vulnerable populations such as pregnant women, infants, and individuals with pre-existing health conditions.

While regulatory measures have been introduced to limit BPA use in consumer products, substitutes such as BPS and BPF are increasingly used without sufficient toxicological evaluation. Given that this study demonstrates similar, if not greater, toxicity for BPS compared to BPA, a more comprehensive risk assessment of these substitutes is necessary. Moreover, microplastics in drinking water and food chains represent a

growing concern, emphasizing the need for stricter policies on plastic waste management and environmental monitoring.

2.2 Ecological and wildlife considerations

Beyond zebrafish, other aquatic and terrestrial organisms are also exposed to bisphenol compounds and microplastics. Fish species consumed by humans, such as tilapia and salmon, may bioaccumulate these contaminants, raising concerns about food safety. Moreover, microplastics have been detected in marine mammals, birds, and terrestrial animals, indicating that their impact extends across ecosystems.

Given that endocrine-disrupting chemicals like bisphenols affect hormone signaling pathways, their presence in the environment could contribute to population declines in wildlife species through reproductive impairment. Future studies should investigate the ecological risks in a broader range of species and explore potential bioindicator organisms for long-term environmental monitoring.

2.3 Regulatory and policy implications

The findings of this study highlight the urgent need for stricter environmental policies on microplastic pollution and endocrine-disrupting chemicals. Currently, regulatory efforts vary across regions, with some countries banning BPA in certain consumer products while others continue to allow its widespread use. International collaboration is essential to establish standardized regulations that address both the direct and indirect effects of these pollutants.

Potential regulatory actions based on this research could include:

Enhanced regulation of bisphenol substitutes: Given the demonstrated toxicity of BPS, regulatory agencies should extend restrictions beyond BPA to include its analogs.

Improved microplastic management: Policies should focus on reducing plastic waste at the source, promoting biodegradable alternatives, and implementing stricter wastewater treatment regulations to capture microplastics before they enter aquatic ecosystems.

Long-term environmental monitoring: Establishing global monitoring networks to track bisphenol and microplastic contamination in water bodies and food chains is crucial for assessing long-term trends and risks.

3. Conclusions

This thesis explored the combined toxicity of bisphenol compounds (BPA and BPS) and microplastics (MP) in zebrafish, with a focus on both adult reproductive health and

transgenerational effects. The research revealed that co-exposure to these environmental pollutants leads to significantly more severe reproductive toxicity than individual exposures, affecting gonadal tissues, hormone levels, and gene expression related to the hypothalamic-pituitary-gonadal-liver (HPGL) axis. Specifically, the study highlighted the negative impact of these pollutants on the expression of genes involved in steroidogenesis, estrogen synthesis, and gamete development, leading to reduced reproductive success. This reproductive dysfunction was observed in both male and female zebrafish, with females exhibiting heightened sensitivity to the toxic effects.

Additionally, the study demonstrated that the toxic effects of co-exposure extend to the F1 generation. Transcriptomic and metabolomic analyses of offspring revealed that parental exposure to BPA, BPS, and MP resulted in altered gene expression and metabolic pathways in offspring, leading to developmental abnormalities and disruptions in key biological processes like lipid metabolism, apoptosis, and calcium signaling. These findings confirm the potential for transgenerational toxicity, where pollutants can induce long-lasting effects that persist across generations.

4. Perspectives

This study provides critical insights into the combined toxicity of bisphenol compounds and microplastics in zebrafish. However, there remain several areas for further investigation. Expanding the scope of research to include a broader range of contaminants, real-world exposure scenarios, and long-term ecological consequences is essential to fully understand the risks posed by these pollutants. The following key areas should be considered in future studies:

(1) Investigating combined pollutant exposures and other emerging contaminants:

In addition to bisphenol analogs and microplastics, numerous other emerging contaminants, such as polyfluoroalkyl substances (PFAS), pharmaceutical residues, and nanoplastics, pose significant environmental risks. Future studies should explore the combined toxic effects of these contaminants, particularly their potential synergistic interactions. Moreover, the role of microplastics as carriers of heavy metals, antibiotics, and persistent organic pollutants (POPs) should be further examined to understand their contribution to bioaccumulation and toxicity in aquatic organisms. Assessing pollutants in isolation may underestimate the ecological risks, as combined exposures significantly enhance bioavailability and toxicity. Regulatory frameworks should incorporate assessments of combined exposures to provide more accurate risk evaluations for aquatic ecosystems.

(2) Long-term and multi-generational studies: Further research is needed to explore the chronic and multi-generational effects of pollutants. While this study demonstrated transgenerational effects in the F1 generation, it remains unclear whether these effects persist, worsen, or diminish in subsequent generations. Future research should extend

to F2 and F3 generations to determine whether epigenetic modifications, developmental abnormalities, or reproductive impairments remain stable or undergo adaptation over time. Understanding these mechanisms is critical for assessing long-term ecological risks.

(3) Expanding research to other aquatic and terrestrial species: The findings in zebrafish serve as a valuable model for aquatic toxicology, but broader investigations are needed to assess the impact of bisphenol compounds and microplastics on other species, including commercially relevant fish, amphibians, and marine mammals. Additionally, exploring the effects of these pollutants in terrestrial ecosystems, such as in drinking water sources, agricultural soils, and airborne microplastics, could provide a more comprehensive understanding of their environmental footprint.

(4) Assessing the relevance of exposure doses and public health implications: One of the critical aspects to consider in future research is the environmental relevance of exposure doses used in laboratory studies. In this study, the concentrations of BPA, BPS, and microplastics were selected to reflect environmentally significant levels based on available literature. However, extrapolating these doses to human exposure requires careful consideration. Humans are primarily exposed to these pollutants through diet (e.g., plastic-packaged food, canned goods, and contaminated water), inhalation, and dermal absorption. Chronic low-dose exposure may still pose risks, especially in vulnerable populations such as pregnant women and infants. Future research should refine exposure models to better mimic realistic contamination levels and routes of exposure. Additionally, incorporating human-relevant metabolic rates and exposure pathways (e.g., ingestion vs. direct water exposure) would enhance the translational value of toxicological findings.

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Appendices

Appendix: List of Publications

Accepted publications (peer reviewed)

1. **Xue, M**., et al. Mechanism analysis of metabolic fatty liver on largemouth bass (*Micropterus salmoides*) based on integrated Lipidomics and proteomics. *Metabolites*, 2022, 12(8), 759. https://doi.org/10.3390/metabo12080759
2. **Xue, M**., et al. Enantioselective behavior of flumequine enantiomers and metabolites' identification in sediment. *Journal of Analytical Methods in Chemistry*, *2022(1), 2184024. https://doi.org/10.1155/2022/2184024*
3. **Xue, M**., et al. Determination of Bisphenol Compounds and the Bioaccumulation after Co-Exposure with Polyethylene Microplastics in Zebrafish. *Toxics*, 2024, 12(10), 702. https://doi.org/10.3390/toxics12100702
4. **Xue, M**., et al. Toxicity of parental co-exposure of microplastic and bisphenol compounds on adult zebrafish: Multi-omics investigations on offspring. *Science of The Total Environment*, 2024, 176897. https://doi.org/10.1016/j.scitotenv.2024.176897

Submitted articles (under review), co-first author

Ming, J., **Xue, M**., et al. Toxic Effects of BPA and BPS Co-Exposure with Microplastics on Zebrafish Health and Transgenerational Impact. *Journal of Hazardous Materials*

Manuscript in preparation

Dessauvages Kenza., Xue, M., et al. A review about microplastic degradation in insects and fish including associated gut bacteria.