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Toxicity mechanisms of polystyrene microplastics in marine mussels revealed by high-coverage quantitative metabolomics using chemical isotope labeling liquid chromatography mass spectrometry

Wei Huang [a,h,1], Xinghuo Wang [b,g,1], Deying Chen [e], Elvis Genbo Xu [d], Xian Luo [c], Jiangning Zeng [a], Tao Huan [f], Liang Li [c,*], Youji Wang [a,b,g,h,**]

[a] Key Laboratory of Marine Ecosystem Dynamics, Second Institute of Oceanography, Ministry of Natural Resources, Hangzhou, China
[b] International Research Center for Marine Biosciences at Shanghai Ocean University, Ministry of Science and Technology, Shanghai 201306, China
[c] Department of Chemistry, University of Alberta, Edmonton, Alberta T6G 2G2, Canada
[d] Department of Biology, University of Southern Denmark, Odense M 5230, Denmark
[e] State Key Laboratory and Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, the First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou 310003, China
[f] Department of Chemistry, University of British Columbia, Vancouver V6T 1Z1, Canada
[g] Key Laboratory of Exploration and Utilization of Aquatic Genetic Resources, Ministry of Education, Shanghai Ocean University, Shanghai 201306, China
[h] Key Laboratory of Satellite Ocean Environment Dynamics, Second Institute of Oceanography, Ministry of Natural Resources, Hangzhou, China

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ABSTRACT

Marine microplastic has become an important environmental issue of global concern due to its wide distribution and harmful impacts. However, there is still insufficient information on the toxicity mechanism of microplastics to marine organisms. In this study, we developed and applied a high-coverage quantitative metabolomics technique to investigate the toxicity mechanisms of the polystyrene microspheres (micro-PS) on marine mussels (*Mytilus coruscus*). A total of 3599 metabolites were quantified, including 163 positively identified metabolites, 318 high-confident putatively identified metabolites, and 2602 mass-matched metabolites from the hemolymph of mussels. Metabolomics analysis indicated that micro-PS disrupted the amino acid metabolism, particularly phenylalanine metabolism, which may lead to oxidative stress and neurotoxicity. Micro-PS at environmentally relevant concentrations induced oxidative stress and immunotoxicity in mussels. After 7 days of recovery, along with the significant clearance of micro-PS by mussels, both metabolite levels and biochemical indicators generally returned to the same level as the control group. Overall, the results showed that microplastics at environmentally-relevant concentrations can cause toxic effects on mussels but these influences are reversible. We envisage the usages of high-coverage metabolomics for investigating the toxicity of various types of microplastics under many different conditions, including those relevant to the marine environment.

1. Introduction

Microplastics are plastic particles with a particle size of less than 5 mm (Arthur et al., 2009; Moore, 2008). In the past two decades, with the massive detection of microplastics in the global ecosystem, microplastic pollution has become a global environmental issue and research hotspot (Law and Thompson, 2014). It is estimated that about five trillion plastic debris are existing in the ocean (Eriksen et al., 2014), causing different socio-economic impacts on various aspects, such as commercial fishery, tourism, shipping, and human health (Thushari and Senevirathna, 2020). Moreover, due to the small particle size and indigestibility of microplastics, it is easy to enter marine organisms (Boerger et al., 2010; Cole et al., 2013; Graham and Thompson, 2009; Li et al., 2015) and transfer between different trophic levels through the food chain

* Corresponding author.
** Corresponding author at: International Research Center for Marine Biosciences at Shanghai Ocean University, Ministry of Science and Technology, Shanghai 201306, China.
E-mail addresses: liang.li@ualberta.ca (L. Li), yj_wang@shou.edu.cn (Y. Wang).
[1] These authors contributed equally to this work.

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(Carbery et al., 2018). These small particles accumulated in organisms can induce physical and biochemical damages, including oxidative stress, digestive tract injury, digestive dysfunction, neurotoxicity, behavioral changes as well as growth inhibition (Ahrendt et al., 2020; Guilhermino et al., 2018; Wang et al., 2020b; Yin et al., 2018; Zhu et al., 2019). However, most studies on the toxicology of microplastics focused on the biochemical response and histological observation of organisms, but the underlying toxicity mechanism of microplastics from a metabolomic perspective remains unclear.

Metabolomics is a useful and versatile tool for exploring unknown toxicity mechanisms of different toxic substances by studying the chemical processes involving metabolites. The metabolome represents the collection of all metabolites, that are the end products of cellular processes, in a biological cell, tissue, organ, or organism (Deng et al., 2017, 2014). Several metabolomic profiling studies have been reported on the investigation of metabolic responses to microplastics using liver tissues (red tilapia, Ding et al., 2020), muscle tissues (zebrafish, Chen et al., 2020b) or gut tissues (zebrafish, Qiao et al., 2019). Some analytical methods, including nuclear magnetic resonance (NMR) and liquid chromatography–mass spectrometry (LC-MS), have been used to analyze the metabolomes of zebrafish (Lu et al., 2016), nematodes (Kim et al., 2020), and corals (Lanctot et al., 2020). However, due to limited sensitivity and/or quantitative accuracy of these methods, only a small number of high-abundance metabolites are quantified in untargeted or targeted metabolomics. The chemical isotope labeling (CIL) LC-MS provides a new opportunity to carry out accurate relative quantification of chemical-group-based submetabolomes with high coverage (Zhao and Li, 2020). This method is usually used for discovering potential metabolite biomarkers of various diseases in the medical field (Chen et al., 2020a), which has never been applied to marine organisms before. In this study, a ^{12}C-/^{13}C-dansylation LC-MS was used to quantify amine/phenol submetabolomes in marine mussels (*Mytilus coruscus*) exposed to polystyrene microspheres (micro-PS) for the first time. In the CIL workflow described in this paper, the individual samples were labeled by ^{12}C-labeling reagent, and the pooled sample generated from mixing all the individual samples was labeled by ^{13}C-labeling reagent. The ^{12}C-/^{13}C- labeled samples were mixed by an equal amount and injected into the mass spectrometer. The dansyl-labeled metabolites were efficiently separated using reserved phase (RP) LC and detected by electrospray ionization (ESI) MS with 10- to 1000-fold increase in detection sensitivity (Zhao and Li, 2020). The CIL workflow afforded the detection of thousands of metabolites from small amounts of mussel hemolymph, thereby allowing a holistic view of the metabolome changes in individual mussels grown under different conditions.

Mussels have been widely used as an ideal indicator for monitoring marine pollution due to their sessile life and they are also one of the most vulnerable species to microplastic pollution (Beyer et al., 2017; Li et al., 2016). The thick shell mussels *Mytilus coruscus* is an important economic shellfish, widely distributed in the East China Sea (Wang et al., 2020b). Contamination of microplastics has been detected in the East China Sea in recent years, and the area around it is considered as one of the world's largest emitters of microplastics (Jambeck et al., 2015; Wang et al., 2020a). This study aims to explore the toxicity mechanism of microplastics in *M. coruscus*. The environmental exposure of microplastics was simulated, and metabolomic changes in hemolymph of mussels exposed to microplastics were quantified by CIL LC-MS for the first time. The bioaccumulation/clearance of microplastics, biochemical, and mRNA responses to microplastics were also investigated. This study provided essential insights into the effects of exposure to plastics. Moreover, the toxicity target of the microplastics was more accurately located through CIL LC-MS.

2. Materials and methods

2.1. Animal and microplastics

Mussels (dry weight 1.50 ± 0.90 g; shell length 7.95 ± 0.32 cm) were collected from Gouqi Island, Zhejiang Province, China. Mussels were transported to the laboratory and acclimated in an aquarium (500 L) filled with filtered seawater for 2 weeks (pH: 8.1 ± 0.1, salinity: 28 ± 1 psu, dissolved oxygen: 7.5 ± 0.2 mg L^{-1}, temperature: 20 ± 1 °C, photoperiod: 14 L/10D). Mussels were fed at 4:00 pm each day with a *Chlorella vulgaris* suspension (5 × 10^4 cells/mL). The filtered seawater was renewed every day and dead mussels were immediately discarded.

Since polystyrene is one of the most commonly used plastic polymers in the world and one of the main components of plastic debris in the ocean (Andrady and Neal, 2009; Browne et al., 2010), fluorescent micro-PS beads (green light, λex: 488 nm, λem: 518 nm, size: 2.0 μm, 1% wt/vol, 5 mL, Shanghai Aladdin Biochemical Technology Co., Ltd., China) were used for this experiment. The micro-PS suspension was dialyzed in 2 L of deionized water using a dialysis bag (MW = 14,000), and the deionized water was changed twice a day for 7 consecutive days to remove additives in the suspension. According to the Micro-Fourier Transform Infrared (μ-FT-IR) analysis, the spectral matching degree of micro-PS was 94% (Fig. S1A). The surface morphology of micro-PS was analyzed by scanning electron microscope (SEM, Hitachi JSM-7500F, Fig. S1B).

Before the exposure experiment, 10 mussels were randomly selected and dissected to extract the digestive tract. The tissue was weighed, put in 10 mL 1 M KOH (1:10), and digested at 65 °C for 48 h (Wu et al., 2020). The potential background fluorescence in the tissue was detected using the enzyme-labeled instrument (Synergy™ Neo2 Multi-Mode Microplate Reader) to ensure that no other fluorescent substances in the digestive tract interfere with fluorescent micro-PS used in the experiments (Lu et al., 2016).

2.2. Experimental design

Four concentrations (0, 10, 10^4, 10^6 particles/L) of micro-PS were set as exposure concentrations, of which 10 particles/L was the environmental concentration reported in the Yangtze River estuary offshore (Zhao et al., 2014), 10^4 particles/L was the approximative measured concentration of microplastics in the Pearl River Estuary (Lam et al., 2020), 10^6 particles/L was the concentration set based on previous toxicological studies (Capolupo et al., 2018; Magni et al., 2018), and clean filtered seawater (0 particles/L micro-PS) was set as the control group.

After the acclimatization period, 360 mussels were randomly selected and placed in 24 glass tanks (30 L, four concentrations, six replicates for each concentration, 15 mussels per tank). The micro-PS stock aqueous suspension (7.03 × 10^8 particles/mL) was diluted and added to tanks to obtain the concentration required for the exposure. Other conditions during the exposure were consistent with the acclimatization period. The filtered seawater and micro-PS were renewed thoroughly daily, then the micro-PS was counted with a hemocytometer (Table S1). After 14 days of exposure, all mussels were transferred to 24 new glass tanks (30 L) for a 7-day recovery experiment. No mortality was observed and the mobility was not affected during the exposure.

2.3. Samples collection

Samples were taken on day 7, 14 (the last day of micro-PS exposure), and 21 (the last day of recovery). At each sampling point, one mussel was randomly selected from each tank (6 mussels per group) and dissected to extract digestive tracts. After tissues were weighed, they were placed in 10 mL 1 M KOH (1:10) and digested at 65 ℃ for 48 h (Wu et al., 2020). The fluorescent micro-PS in tissues was quantified by the enzyme-labeled instrument (Synergy™ Neo2 Multi-Mode Microplate

Reader, Lu et al., 2016). A second mussel from each tank (6 mussels per group) was first taken for extracting 2 mL hemolymph from the posterior adductor muscle with a 2 mL syringe, and then dissected to sample digestive glands. The digestive gland and hemolymph samples were immediately placed in liquid nitrogen, then all samples were stored at − 80 °C for subsequent biochemical measurements and metabolomics analysis, respectively.

2.4. Biochemical measurement

The weight of digestive glands was measured individually for each mussel (100–120 mg), and the normal saline was added with a proportion of weight (g): volume (mL) = 1:9. Then homogenization was carried out on ice water bath. After centrifuging at 2500 rpm for 10 min (Centrifuge 5810R, Eppendorf, Hamburg, Germany), the supernatant was taken for subsequent detections of biochemical parameters. Reactive oxygen species (ROS) level, lipid hydroperoxide (LPO) level, malondialdehyde (MDA) level, acid phosphatase (ACP) activity, and alkaline phosphatase (ALP) activity were measured using commercially available kits (Jiancheng Bioengineering Institute, Nanjing, China).

According to the method of Gu et al. (2020b), the total mRNA was extracted from digestive glands (50 mg) for real-time quantitative PCR analysis (ABI 7500 Real-Time PCR System, Applied Biosystems, Singapore) of heat shock proteins 70 and 90 mRNA expression (Hsp70 and Hsp90, Table S2).

2.5. Metabolomic analysis

One 60 μL aliquot of each mussel hemolymph was used to extract metabolites using pre-cold methanol with a 3:1 ratio (vol:vol). After 10 s mixing with vortex, and the mixture was transferred to − 20 °C refrigerator, then the suspension was centrifuged at 13,500 rpm at room temperature for 15 min. Finally, the supernatant was transferred to another 0.5 mL plastic vial and dried using a SpeedVac vacuum concentrator. The combination of sample drying and low-temperature storage were important steps for maximizing the stability of metabolites. Afterwards, dried mussel extracts were re-dissolved in 40 μL liquid chromatography–mass spectrometry (LC-MS) grade water. Then, a pooled sample was prepared by mixing a 15-μL of aliquot of an individual sample from a total of 72 samples (4 groups: 0, 10, 10^4 and 10^6 particles/L micro-PS; 3 time points: 7, 14 and 21 days; 6 replicates), which served as quality control (QC) and the reference sample for relative quantification (see below).

^{12}C-dansyl chloride (Dns-Cl) was used to label individual samples and ^{13}C−Dns-Cl obtained from www.novamt.com was used to label the pooled sample (Note S1). Sample-wise normalization was conducted by measuring the total concentration of Dns-labeled amine/phenol-containing metabolites using liquid chromatography with ultraviolet (LC-UV) at 338 nm (Note S1, Guo and Li, 2010). Each ^{12}C-labeled individual sample was then mixed with the ^{13}C-labeled pooled sample in 1:1 mol ratio.

The labeled sample was analyzed using UltiMate3000 UHPLC (Thermo Scientific, MA) combined with the Impact II Quadrupole Time-of-flight (QTOF) mass spectrometer (Bruker, Billerica, MA, Note S1). Only positive ion detection was used as dansyl labeled metabolites were preferentially ionized in positive mode. A QC sample, prepared by mixing equal moles of ^{12}C- and ^{13}C-labeled pooled samples, was run after every 20 sample runs.

In CIL LC-MS, dansyl labeled metabolites are detected in the form of peak pairs. Metabolite peak pair extraction, sample-wise alignment, and missing value retrieval were conducted by using IsoMS Pro (www.novamt.com). Metabolite identification was carried out using a three-tier approach reported previously (Zhao et al., 2019).

2.6. Statistical analysis

Statistics were performed by SPSS 25 (SPSS Inc., Chicago, IL, USA). The homogeneity of the data was tested by Levene's test, and the normal distribution of the data was detected by Shapiro-Wilk's test. The effect of micro-PS on mussels of the different groups at each sampling date were determined through one-way ANOVA followed by Tukey's HSD tests. Student's *t*-test was carried out for analyzing the significant effects between sampling day 7 and 14 on the biochemical parameters at a fixed micro-PS concentration. The results are expressed as the mean ± standard deviation (means ± SD), and $p < 0.05$ indicates a significant difference between the groups. Metabolomics data were completed by SIMCA-P+ (version 12.0; Umetrics, Umea, Sweden) and MetaboAnalyst (www.metaboanalyst.ca). OriginPro 2020 (OriginLab Inc., USA) was used to analyze the correlation between biochemical indicators and metabolites, as well as to draw heat map.

3. Results and discussion

3.1. Micro-PS accumulation and clearance

The size of microplastics is within the ideal feeding range of a variety of marine organisms, thus these particles are easy to be ingested by mussels and cause adverse effects (Wright et al., 2013). As representative benthic filter feeders, mussels can effectively accumulate micropollutants in seawater (Beyer et al., 2017). Moreover, compared with other tissues, the accumulation of microplastics in the digestive system is significantly higher (Avio et al., 2015; Kolandhasamy et al., 2018). In this study, we exposed mussels to three micro-PS concentrations for 14 days. At the two high-concentration exposures (10^4 and 10^6 particles/L), the accumulation of micro-PS in the digestive tract increased significantly (Table 1) but no micro-PS accumulation was detected in the control and 10 particles/L micro-PS groups. The maximum concentration of micro-PS accumulated in the digestive tract was 0.926 μg/mg. These results indicated the bioaccumulation potential of microplastics in mussels. The microplastics ingested by the organism can cause physical damage such as scratches, perforations, and blockages in the digestive tract (Voelker et al., 2017; Wright et al., 2013). Moreover, microplastics caused decreased digestive enzyme activities and changes in intestinal microbes (Gu et al., 2020a; Wang et al., 2020b). Interestingly, after a 7-day recovery period (on day 21), the accumulation of micro-PS in the two treatments with high microplastic concentrations significantly decreased (Table 1). This indicated that microplastics can be cleared out from the body by mussels in clean water.

3.2. Oxidative stress and immune responses

During the micro-PS exposure, the increase of ROS was accompanied by the increase of lipid peroxidation index (MDA and LPO). These significant upward trends were only observed in the 10^4 and 10^6 particles/L micro-PS groups compared with the control group, and there was no significant difference between the 10 particles/L micro-PS group and the control group (Fig. 1). Similarly, previous studies have shown that microplastics can cause significant effects on the oxidation state of

Table 1
The accumulation of microplastics in the digestive tract of mussels. Different lowercase letters represent significant differences between different sampling days under the same polystyrene microspheres (micro-PS) concentrations ($p < 0.05$).

Group	Day 7 (10^{-3} μg/mg)	Day 14 (10^{-3} μg/mg)	Day 21 (10^{-3} μg/mg)
10^4 particles/L	9.363 ± 0.372^{b}	11.587 ± 0.437^{a}	7.083 ± 0.454^{c}
10^6 particles/L	742.373 ± 24.223^{b}	926.110 ± 25.405^{a}	15.713 ± 1.870^{c}

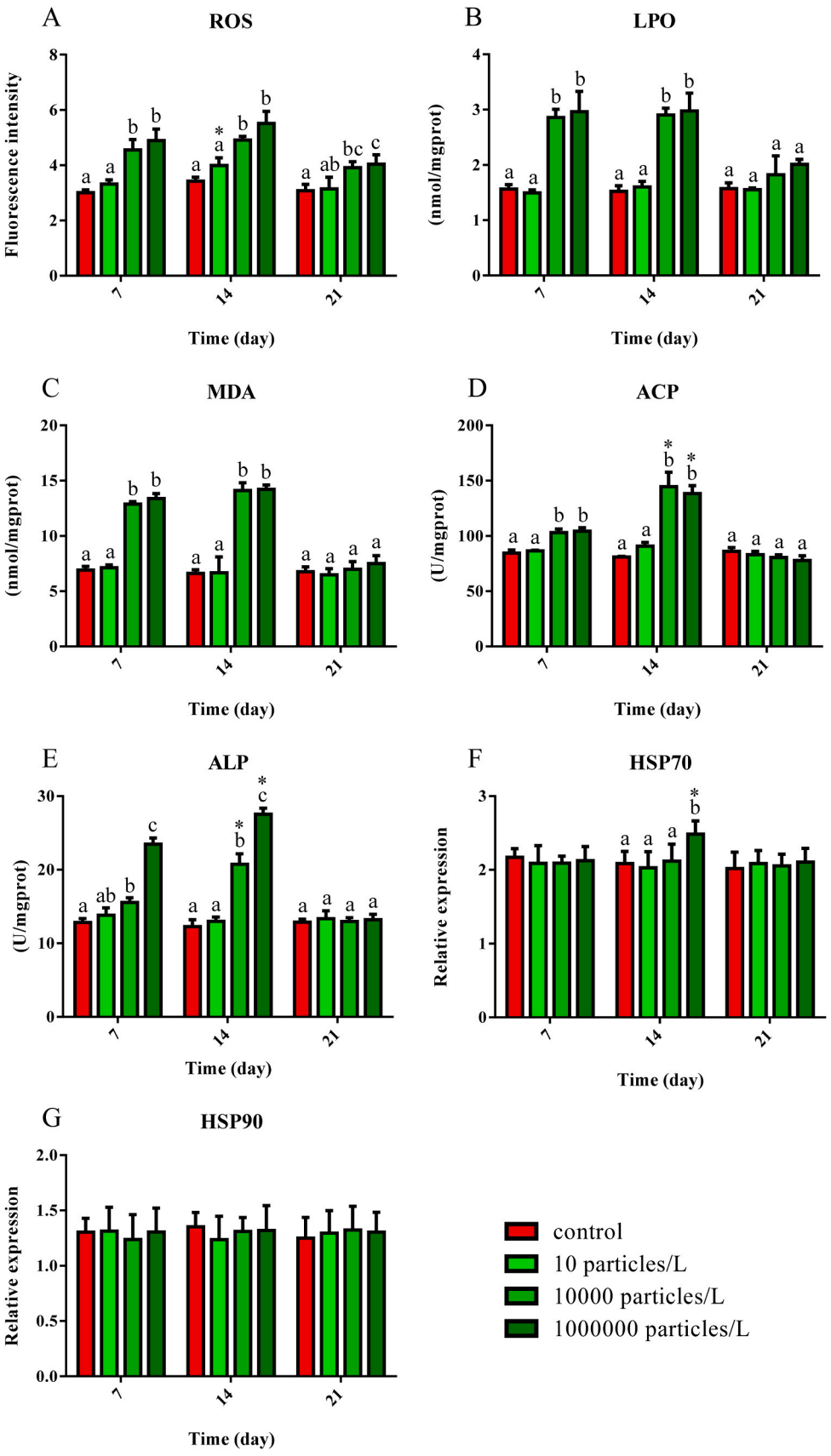

Fig. 1. Reactive oxygen species (ROS) level (A), lipid hydroperoxide (LPO) level (B), malondialdehyde (MDA) level (C), acid phosphatase (ACP) activity (D), alkaline phosphatase (ALP) activity (E), heat shock proteins 70 (Hsp70) and Hsp90 (F and G) expression level in digestive gland of mussels exposed to different polystyrene microspheres (micro-PS) concentrations (0, 10, 10,000 and 1,000,000 particles/L). Different lowercase letters represent significant difference ($p < 0.05$) between different micro-PS concentrations (e.g., there is a significant difference between "a" and "b", there is no significant difference between "a" and "ab", and there is no significant difference between "b" and "ab"); * indicates significant difference ($p < 0.05$) between different time under the micro-PS concentrations during the exposure period.

bivalve mollusks, including changes in peroxisome proliferation and antioxidant systems (Avio et al., 2015; Magni et al., 2018), and upregulation of oxidative stress-related genes (Détrée and Gallardo-Escárate, 2017). However, during the recovery period, these significant differences disappeared (Fig. 1), suggesting a causal association between bioaccumulation and oxidative stress.

As for immune parameters, 10^4 and 10^6 particles/L micro-PS significantly increased the ACP and ALP activities of mussels, and they also increased significantly with the increase of exposure time (Fig. 1). ACP and ALP are biomarkers for immune responses to environmental pollutants (Mazorra et al., 2002), for example, as indicators of stimulated phagocytosis (Chi et al., 2017). The exposure of micro-PS significantly increased the activities of ACP and ALP, suggesting immune responses of mussels under the pressure of micro-PS to resist the toxicity of micro-PS. This is consistent with our previous study that microplastics significantly activate another immune indicator (lysozyme) of mussels (Wang et al., 2020b). Similar to the trend of oxidative stress indicators, during the recovery period, both ACP and ALP activities returned to the same level as the control group. These results further implied the causal relationship between micro-PS accumulation and the measured effects.

The mRNA expression of heat shock protein Hsp70 was significantly upregulated after 14 days of exposure to the 10^6 particles/L micro-PS, and it also increased significantly with the increase of exposure time (Fig. 1). Heat shock proteins play a vital role in protecting cells from damage and repairing damaged proteins (Gao et al., 2007; Song et al., 2006). However, further studies are needed to understand protein damages by microplastic exposure and the protective responses of mussels.

Although mussels showed a significant response to microplastics in terms of oxidative stress and immunotoxicity, these effects only occurred under 10^4 and 10^6 particles/L micro-PS. Under environmental concentration of 10 particles/L micro-PS, no significant changes were observed, suggesting negligible acute toxicity and low environmental risk of the tested microplastics to mussels in short term.

3.3. Metabolomic analysis

In total, 3599 serum metabolites in 24 samples were detected and quantified. Metabolite identification was carried out using a three-tier approach and the results are shown in Table S3. There were 163 metabolites positively identified and 318 putatively identified with high-confidence. There were 574, 1460, and 568 peak pairs matched to the zero-, one-, and two-reaction MyCompoundID libraries (Li et al., 2013), respectively. In other words, out of the 3599 detected peak pairs, 3083 (85.7%) were either identified or mass-matched. The high coverage enabled a global examination of the micro-PS effects on the mussel metabolome.

Fig. S2 shows the principal component analysis (PCA) score plot projecting the 12 groups and the QC samples. The QCs were clustered together tightly, indicating high instrumental stability during the data collection period. The corresponding partial least-squares discriminant analysis (PLS-DA) score plot is shown in Fig. 2. On day 7 (Fig. 2A), the three micro-PS treatments overlapped slightly but separated from the control. On day 14 (Fig. 2B), all micro-PS treatments overlapped significantly, and their separation from the control was more significant. After a 7-day recovery period (Fig. 2C), there were significant overlaps between the micro-PS treatment groups and control. Table S4 lists the number of significant metabolites found in the binary comparison of two groups subjected to different micro-PS concentrations (fold change $\geq$ 1.2, false discovery rate $<$ 0.05). Consistent with the multivariate

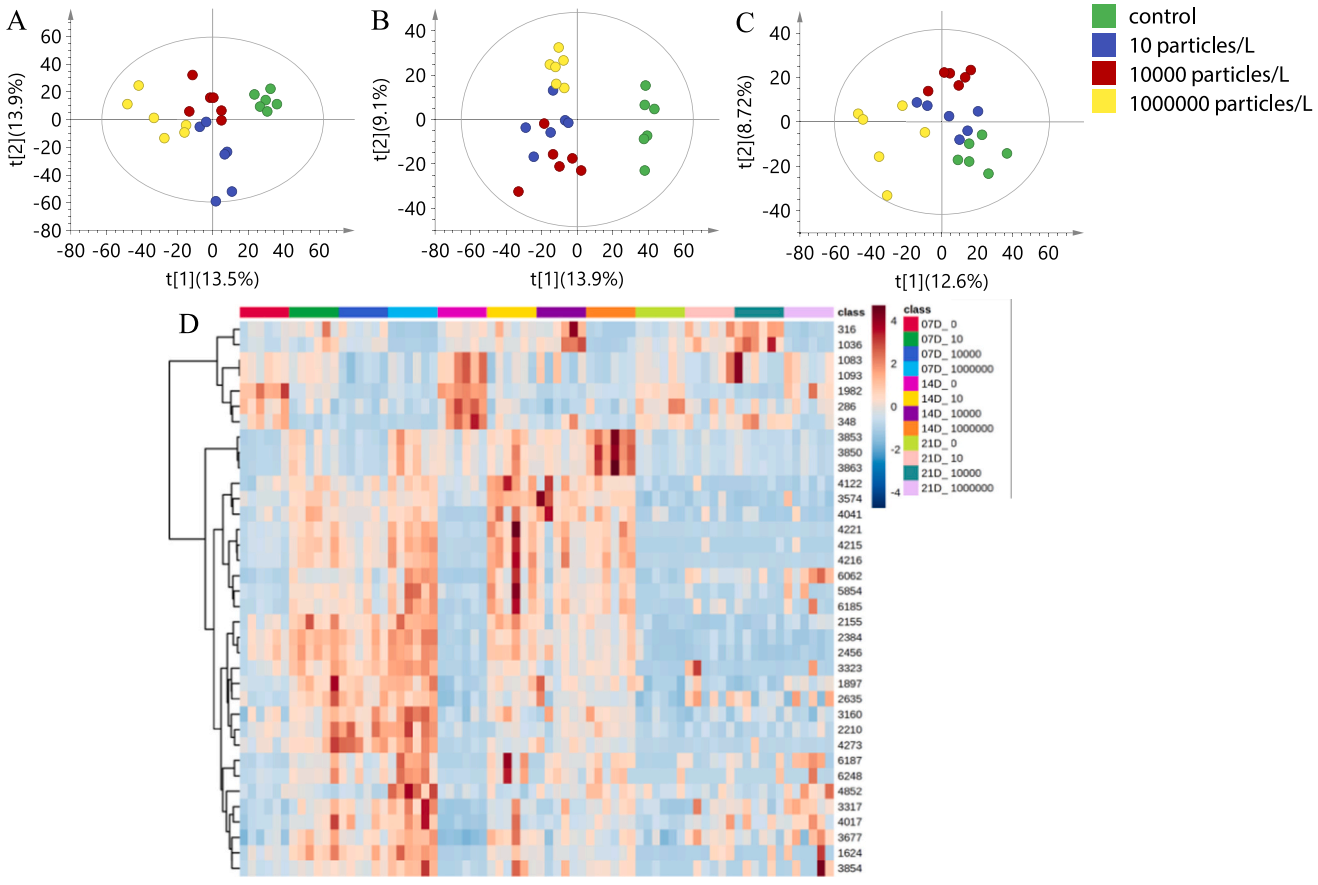

Fig. 2. Partial least-squares discriminant analysis (PLS-DA) results obtained from liquid chromatography–mass spectrometry (LC-MS) data of metabolites extracted from mussel hemolymph exposed to micro-PS (A: day 7, B: day 14, C: day 21). Heat map of 36 significant metabolites identified (D).

analysis, there were no significant differences among treatments during the recovery period, suggesting overall similar metabolome profiles between the control and treatment groups during the recovery period.

In addition, we compared the significant metabolites on day 7 and 14 between the high micro-PS concentration group and the control. There were 36 metabolites in common (Table S5, Fig. 2D). Among them, 7 metabolites were positively identified and putatively identified with high-confidence (Table S6). Next, we investigated whether changes in

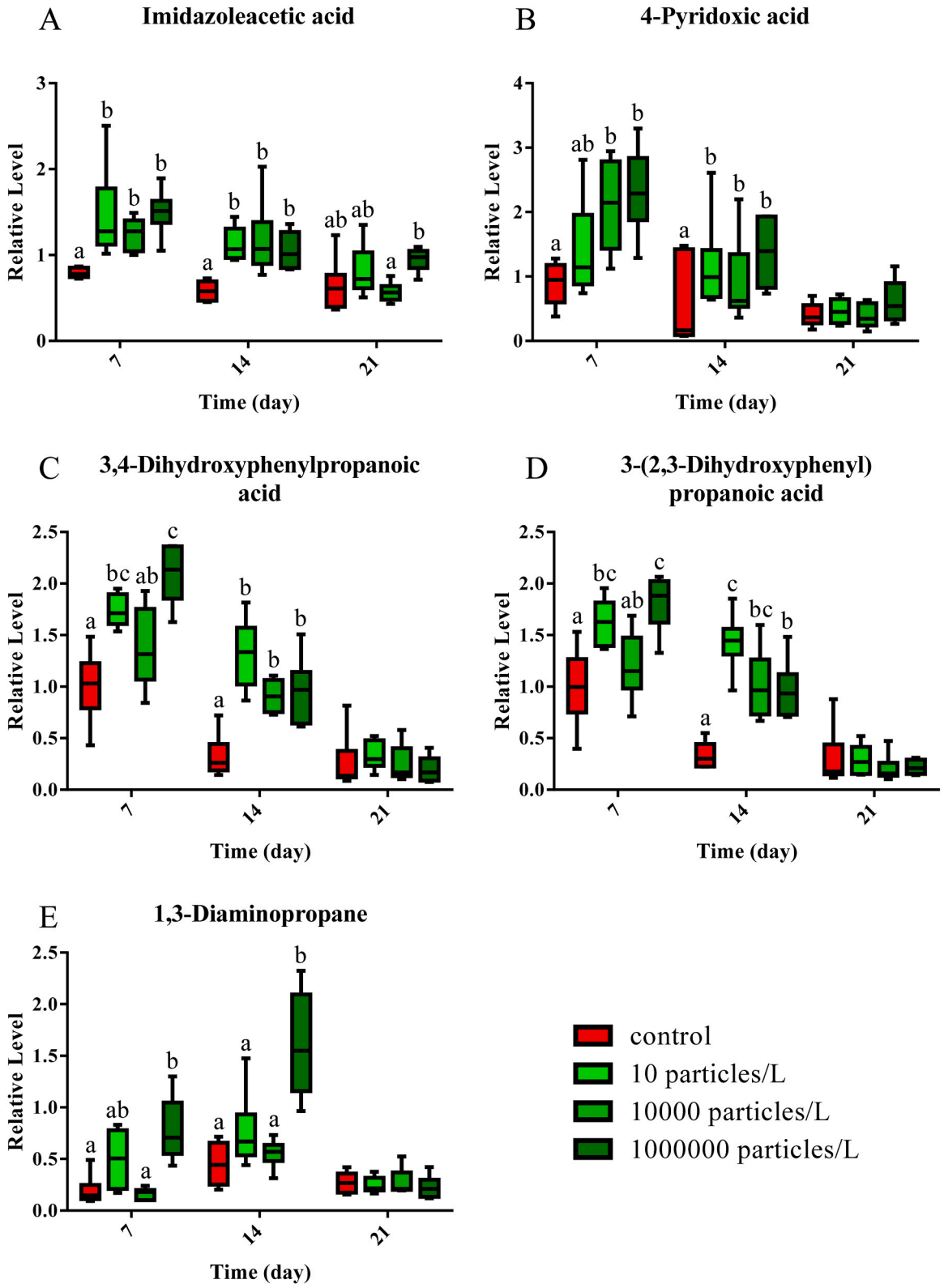

Fig. 3. Changed in the metabolites of mussel hemolymph exposed to micro-PS. Different lowercase letters represent significant difference ($p < 0.05$) between different micro-PS concentrations (e.g., there is a significant difference between "a" and "b", there is no significant difference between "a" and "ab", and there is no significant difference between "b" and "ab").

the levels of metabolites follow the same pattern of changes as a function of increasing micro-PS concentrations. Except for nopalinic acid and beta-alanyl-L-lysine (Fig. S3), the other 5 metabolites all followed the same pattern of changes as the micro-PS concentrations increased (Fig. 3). It is worth noting that there were no significant effects on oxidative stress or immune-related indicators under the environmental concentration of 10 particles/L micro-PS, however, the more sensitive metabolomics analyses identified significant changes of 4 metabolites (Imidazoleacetic acid, 4-Pyridoxic acid, 3,4-Dihydroxyphenylpropanoic acid, 3-(2,3-Dihydroxyphenyl) propanoic acid) at the low environmentally relevant concentration of micro-PS (10 particles/L).

To further understand the acting mechanisms of micro-PS on mussels, the pathways of these 5 metabolites were analyzed. The results indicated that they were involved in histidine metabolism, tyrosine metabolism, phenylalanine metabolism, beta-alanine metabolism, and vitamin B6 metabolism pathways (Table 2).

Our previous study found if microplastics enter the digestive system of mussels, they can cause changes in protease activity (Wang et al., 2020b). This may be the cause of the disorder of amino acid metabolism. Moreover, studies have shown that high levels of tyrosine can induce oxidative stress (Sgaravatti et al., 2008). Phenylalanine and its metabolites can cause an increase in lipid peroxidation (Fernandes et al., 2010). Beta-alanine can increase the level of ROS and cause oxidative damage to cells (Gemelli et al., 2018), which is consistent with the results of this experiment. Our correlation analysis showed that oxidative stress indicators and the changing trend of differential metabolites were highly correlated with ROS levels (Fig. 4). It indicated that the disorder of the metabolism of these amino acids may be the key process that causes oxidative stress in mussels. Phenylalanine is a biomarker of neurobehavioral effects and a precursor of tyrosine (Elie et al., 2015). They can be further converted into monoamine neurotransmitters (Fernstrom and Fernstrom, 2007). Previous studies have also confirmed the neurotoxicity of microplastics to organisms, for example, the inhibition of acetyl cholinesterase (AChE) activity in red tilapia (*Oreochromis niloticus*, Ding et al., 2020). Interestingly, a recent study found that in wild fish, LPO and AChE in microplastic-polluted fish samples were significantly higher (Barboza et al., 2020). The author indicated that lipid peroxidation may cause the rupture of the vesicle membrane containing acetylcholine, which led to an increase in the release of neurotransmitters into cholinergic synaptic clefts and consequently induced AChE production as a compensatory strategy (Barboza et al., 2020). Therefore, the level of phenylalanine is a potential indicator for evaluating the neurotoxicity of microplastics. Vitamin B6 can also directly react with the peroxy radicals to scavenge radicals and inhibit lipid peroxidation (Bilski et al., 2000; Kannan and Jain, 2004; Keles et al., 2010). More researches have started to apply metabolomics to understand the molecular mechanisms of microplastic toxicity, confirming the effects of microplastics on the metabolism of amino acids. For example, Cappello et al. (2021) used protonic Nuclear Magnetic Resonance (^{1}H NMR)-based metabolomics to explore the toxicity mechanism of microplastics to Mediterranean mussel *Mytilus galloprovincialis*, and found that the metabolism of amino acids (glycine, alanine, tyrosine, etc.) was significantly disturbed. Teng et al. (2021) explored the toxicity mechanism of microplastics to oyster *Crassostrea gigas* through ultrahigh-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS), and found that microplastics can cause amino acid metabolism (arginine and proline metabolism, beta-alanine metabolism, histidine metabolism, tryptophan metabolism and glycine, serine and threonine metabolism) disorders in the digestive glands of oyster.

Table 2
The shared metabolites and their pathways among different mussel groups.

NovaMT Library No.	Metabolite	Pathway
AAA00825000	Imidazoleacetic acid	Histidine metabolism
AAA01610000	4-Pyridoxic acid	Vitamin B6 metabolism
AAA00916000	3,4-Dihydroxyphenylpropanoic acid	Tyrosine metabolism
AAA00929000	3-(2,3-Dihydroxyphenyl) propanoic acid	Phenylalanine metabolism
AAA00641000	1,3-Diaminopropane	beta-Alanine metabolism

Tyrosine, phenylalanine, histidine, and beta-alanine metabolism are involved in the tricarboxylic acid cycle (TCA cycle, Fig. 5). The TCA cycle is the center of the interconnected cycle of energy transmission (Gibala et al., 2000). Changed in these metabolic pathways can interfere with the TCA cycle and affect the energy supply of cells, and it will further affect immunity, metabolism, and growth activities. The high correlation between immune indicators and the changing trend of differential metabolites generated this hypothesis (Fig. 5). In a previous study, Détrée and Gallardo-Escárate (2018) explored the toxicity of microplastics in the edible mussel *Mytilus galloprovincialis* via transcriptomics analyses, and found that microplastics can lead to the regulation of immune and stress-related processes and affect energy metabolism. Sussarellu et al. (2016) explored the transcriptomic and proteomic responses of oyster *Crassostrea gigas* after microplastic exposure, and found that microplastics can interfere with energy absorption and distribution.

Although micro-PS caused significant effects and responses of the antioxidant system and immune system of mussels, it is worth noting that after 7 days of depuration, most of the different metabolite levels and biochemical parameters were recovered. We for the first time reported that environmental concentrations of micro-PS significantly changed the metabolome profiles by high-coverage quantitative metabolomics analyses but recovery of metabolite levels was also observed after depuration. It may indicate that microplastics do not have serious and irreversible toxicity to organisms. However, it is still unknown whether such recovery can occur in the natural environment, and we also don't know the metabolomic responses to environmental microplastics that coexist with other chemical pollutants (Gandara e Silva et al., 2016).

Overall, a new CIL LC-MS-based metabolomics technology combined with traditional biochemical methods was used to study the toxicity mechanisms of micro-PS on mussels. Micro-PS caused significant changes in the metabolism of tyrosine, phenylalanine, histidine, beta-alanine, and vitamin B6 metabolism in the hemolymph of mussels, and also affected the antioxidant and immune parameters. In addition, micro-PS in environmentally relevant concentrations (10 and 10^4 particles/L) caused significant differences in metabolite levels. During the recovery period, most indicators were restored. This work highlighted that the combination of metabolomics and biochemical methods can advance understanding of the toxicity pathways of environmental pollutants. Inspired by the current work, we will further apply this metabolomics approach to examine the toxic effects of other types of micro-PS with varying particle sizes, size distributions, polymer compositions, and modified particle surfaces. We will also extend the current coverage of the amine/phenol submetabolome to include other chemical-group-based submetabolomes (e.g., acid, carbonyl, and hydroxyl submetabolomes) for a global metabolome profiling of potential toxicity pathways. In order to reveal other potentially distributed biological functions, the current library of standards needs to be expanded to improve the identification of more metabolites in the future. Finally, the global metabolome profiling results provide valuable bases to generate evidence-based hypotheses on the toxicity mechanism of microplastic. Future studies to include different polymer types and morphological types of micro-PS, as well as cellular and targeted tissue models, are needed to detail the mechanistic toxicity of microplastic.

CRediT authorship contribution statement

Wei Huang: Conceptualization, Writing - review & editing, Project

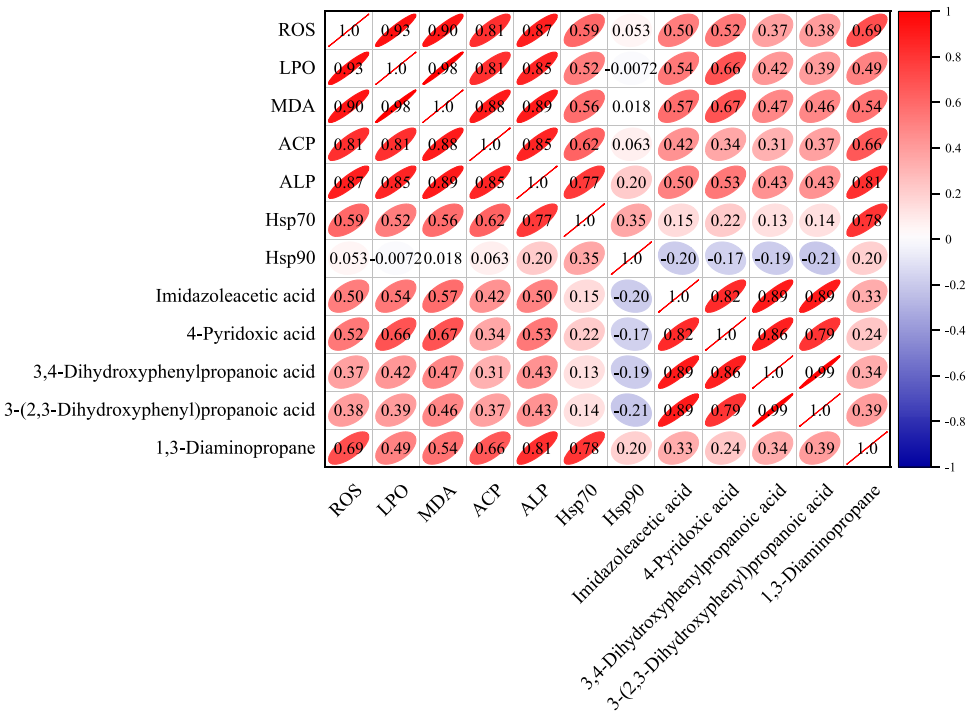

Fig. 4. Heat map of the correlation between biochemical indicators and different metabolites. The numbers indicate Pearson correlation coefficient. Red represents a positive correlation and blue represents a negative correlation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

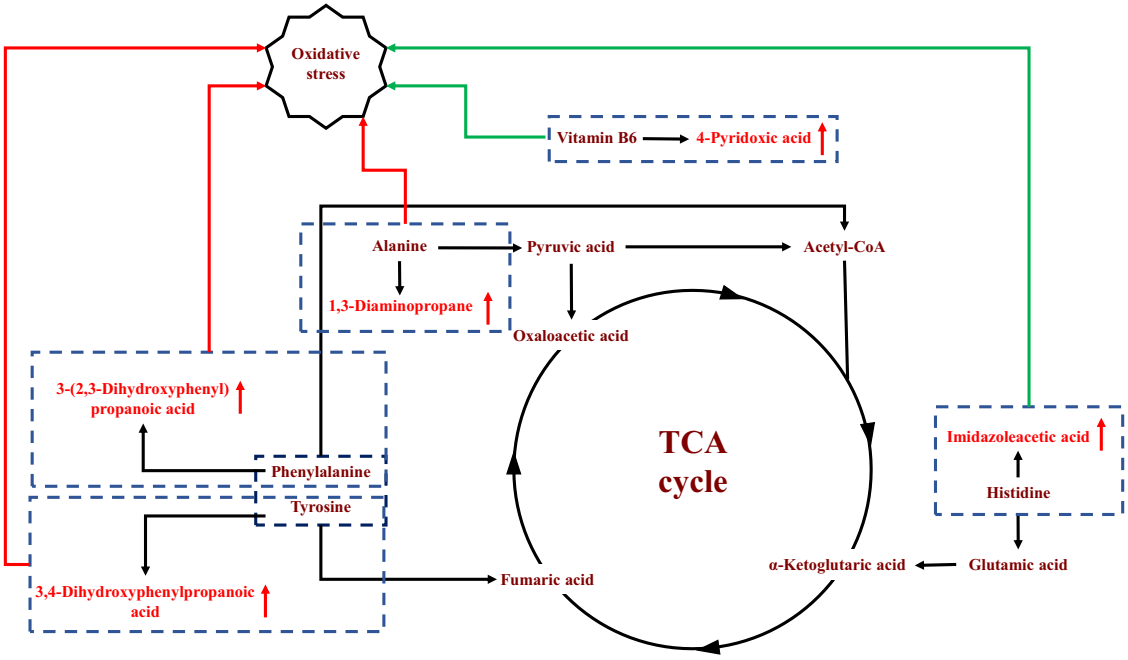

Fig. 5. Metabolic network diagram of mussel hemolymph biomarkers induced by micro-PS.

administration, Supervision. **Xinghuo Wang**: Conceptualization, Investigation, Writing - original draft. **Deying Chen**: Methodology, Software, Writing - review & editing. **Elvis Genbo Xu**: Writing - review & editing, Visualization. **Xian Luo**: Writing - review & editing. **Jiangning Zeng**: Writing - review & editing. **Tao Huan**: Writing - review & editing. **Liang Li**: Writing - review & editing, Resources. **Youji Wang**: Conceptualization, Writing - reviewing & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2021.126003.

References

Ahrendt, C., Perez-Venegas, D.J., Urbina, M., Gonzalez, C., Echeveste, P., Aldana, M., Pulgar, J., Galbán-Malagón, C., 2020. Microplastic ingestion cause intestinal lesions in the intertidal fish Girella laevifrons. Mar. Pollut. Bull. 151, 110795.

Andrady, A.L., Neal, M.A., 2009. Applications and societal benefits of plastics. Philos. Trans. R Soc. B Biol. Sci. 364, 1977–1984.

Arthur, C.A., Baker, J., Bamford, H., 2009. Proceedings of the International Research Work-shop on the Occurrence, Effects, and Fate of Microplastic Marine Debris, September 9–11. NOAA Technical Memorandum NOS-OR&R-30, 2009, Washington.

Avio, C.G., Gorbi, S., Milan, M., Benedetti, M., Fattorini, D., d'Errico, G., Pauletto, M., Bargelloni, L., Regoli, F., 2015. Pollutants bioavailability and toxicological risk from microplastics to marine mussels. Environ. Pollut. 198, 211–222.

Barboza, L.G.A., Lopes, C., Oliveira, P., Bessa, F., Otero, V., Henriques, B., Raimundo, J., Caetano, M., Vale, C., Guilhermino, L., 2020. Microplastics in wild fish from North East Atlantic Ocean and its potential for causing neurotoxic effects, lipid oxidative damage, and human health risks associated with ingestion exposure. Sci. Total Environ. 717, 134625.

Beyer, J., Green, N.W., Brooks, S., Allan, I.J., Ruus, A., Gomes, T., Bråte, I.L.N., Schøyen, M., 2017. Blue mussels (*Mytilus edulis* spp.) as sentinel organisms in coastal pollution monitoring: a review. Mar. Environ. Res. 130, 338–365.

Bilski, P., Li, M.Y., Ehrenshaft, M., Daub, M.E., Chignell, C.F., 2000. Vitamin B-6 (pyridoxine) and its derivatives are efficient singlet oxygen quenchers and potential fungal antioxidants. Photochem. Photobiol. 71, 129–134.

Boerger, C.M., Lattin, G.L., Moore, S.L., Moore, C.J., 2010. Plastic ingestion by planktivorous fishes in the North Pacific Central Gyre. Mar. Pollut. Bull. 60, 2275–2278.

Browne, M.A., Galloway, T.S., Thompson, R.C., 2010. Spatial patterns of plastic debris along estuarine shorelines. Environ. Sci. Technol. 44, 3404–3409.

Capolupo, M., Franzellitti, S., Valbonesi, P., Sanz Lanzas, C., Fabbri, E., 2018. Uptake and transcriptional effects of polystyrene microplastics in larval stages of the Mediterranean mussel *Mytilus galloprovincialis*. Environ. Pollut. 241, 1038–1047.

Cappello, T., De Marco, G., Oliveri Conti, G., Giannetto, A., Ferrante, M., Mauceri, A., Maisano, M., 2021. Time-dependent metabolic disorders induced by short-term exposure to polystyrene microplastics in the Mediterranean mussel *Mytilus galloprovincialis*. Ecotoxicol. Environ. Saf. 209, 111780.

Carbery, M., O'Connor, W., Palanisami, T., 2018. Trophic transfer of microplastics and mixed contaminants in the marine food web and implications for human health. Environ. Int. 115, 400–409.

Chen, D., Han, W., Huan, T., Li, L., Li, L., 2020a. Effects of freeze-thaw cycles of blood samples on high-coverage quantitative metabolomics. Anal. Chem. 92, 9265–9272.

Chen, Q., Lackmann, C., Wang, W., Seiler, T.-B., Hollert, H., Shi, H., 2020b. Microplastics lead to hyperactive swimming behaviour in adult zebrafish. Aquat. Toxicol. 224, 105521.

Chi, C., Giri, S.S., Jun, J.W., Kim, H.J., Kim, S.W., Yun, S., Park, S.C., 2017. Effects of algal toxin okadaic acid on the non-specific immune and antioxidant response of bay scallop (*Argopecten irradians*). Fish Shellfish Immunol. 65, 111–117.

Cole, M., Lindeque, P., Fileman, E., Halsband, C., Goodhead, R., Moger, J., Galloway, T. S., 2013. Microplastic ingestion by zooplankton. Environ. Sci. Technol. 47, 6646–6655.

Deng, Y., Zhang, Y., Zhang, R., Wu, B., Ding, L., Xu, K., Ren, H., 2014. Mice in vivo toxicity studies for monohaloacetamides emerging disinfection byproducts based on metabolomic methods. Environ. Sci. Technol. 48, 8212–8218.

Deng, Y., Zhang, Y., Lu, Y., Lu, K., Bai, H., Ren, H., 2017. Metabolomics evaluation of the in vivo toxicity of bromoacetonitriles: one class of high-risk nitrogenous disinfection byproducts. Sci. Total Environ. 579, 107–114.

Détrée, C., Gallardo-Escárate, C., 2017. Polyethylene microbeads induce transcriptional responses with tissue-dependent patterns in the mussel *Mytilus galloprovincialis*. J. Molluscan Stud. 83, 220–225.

Détrée, C., Gallardo-Escárate, C., 2018. Single and repetitive microplastics exposures induce immune system modulation and homeostasis alteration in the edible mussel *Mytilus galloprovincialis*. Fish Shellfish Immunol. 83, 52–60.

Ding, J., Huang, Y., Liu, S., Zhang, S., Zou, H., Wang, Z., Zhu, W., Geng, J., 2020. Toxicological effects of nano- and micro-polystyrene plastics on red tilapia: are larger plastic particles more harmless? J. Hazard. Mater. 396, 122693.

Elie, M.R., Choi, J., Nkrumah-Elie, Y.M., Gonnerman, G.D., Stevens, J.F., Tanguay, R.L., 2015. Metabolomic analysis to define and compare the effects of PAHs and oxygenated PAHs in developing zebrafish. Environ. Res. 140, 502–510.

Eriksen, M., Lebreton, L.C.M., Carson, H.S., Thiel, M., Moore, C.J., Borerro, J.C., Galgani, F., Ryan, P.G., Reisser, J., 2014. Plastic pollution in the world's oceans: more than 5 trillion plastic pieces weighing over 250,000 tons afloat at sea. PLoS One 9, 111913.

Fernandes, C.G., Leipnitz, G., Seminotti, B., Amaral, A.U., Zanatta, Â., Vargas, C.R., Dutra Filho, C.S., Wajner, M., 2010. Experimental evidence that phenylalanine provokes oxidative stress in hippocampus and cerebral cortex of developing rats. Cell. Mol. Neurobiol. 30, 317–326.

Fernstrom, J.D., Fernstrom, M.H., 2007. Tyrosine, phenylalanine, and catecholamine synthesis and function in the brain. J. Nutr. 137, 1539S–1547S.

Gandara e Silva, P.P., Nobre, C.R., Resaffe, P., Seabra Pereira, C.D., Gusmao, F., 2016. Leachate from microplastics impairs larval development in brown mussels. Water Res. 106, 364–370.

Gao, Q., Song, L., Ni, D., Wu, L., Zhang, H., Chang, Y., 2007. cDNA cloning and mRNA expression of heat shock protein 90 gene in the haemocytes of Zhikong scallop Chlamys farreri. Comp. Biochem. Physiol. Part B Biochem. Mol. Biol. 147, 704–715.

Gemelli, T., de Andrade, R.B., Rojas, D.B., Zanatta, Â., Schirmbeck, G.H., Funchal, C., Wajner, M., Dutra-Filho, C.S., Wannmacher, C.M.D., 2018. Chronic exposure to β-alanine generates oxidative stress and alters energy metabolism in cerebral cortex and cerebellum of wistar rats. Mol. Neurobiol. 55, 5101–5110.

Gibala, M.J., Young, M.E., Taegtmeyer, H., 2000. Anaplerosis of the citric acid cycle: role in energy metabolism of heart and skeletal muscle. Acta Physiol. Scand. 168, 657–665.

Graham, E.R., Thompson, J.T., 2009. Deposit- and suspension-feeding sea cucumbers (*Echinodermata*) ingest plastic fragments. J. Exp. Mar. Biol. Ecol. 368, 22–29.

Gu, H., Wang, S., Wang, X., Yu, X., Hu, M., Huang, W., Wang, Y., 2020a. Nanoplastics impair the intestinal health of the juvenile large yellow croaker *Larimichthys crocea*. J. Hazard. Mater. 397, 122773.

Gu, H., Wei, S., Hu, M., Wei, H., Wang, X., Shang, Y., Li, La, Shi, H., Wang, Y., 2020b. Microplastics aggravate the adverse effects of BDE-47 on physiological and defense performance in mussels. J. Hazard. Mater. 398, 122909.

Guilhermino, L., Vieira, L.R., Ribeiro, D., Tavares, A.S., Cardoso, V., Alves, A., Almeida, J.M., 2018. Uptake and effects of the antimicrobial florfenicol, microplastics and their mixtures on freshwater exotic invasive bivalve Corbicula fluminea. Sci. Total Environ. 622, 1131–1142.

Guo, K., Li, L., 2010. High-performance isotope labeling for profiling carboxylic acid-containing metabolites in biofluids by mass spectrometry. Anal. Chem. 82, 8789–8793.

Jambeck, J.R., Geyer, R., Wilcox, C., Siegler, T.R., Perryman, M., Andrady, A., Narayan, R., Law, K.L., 2015. Plastic waste inputs from land into the ocean. Science 347, 768–771.

Kannan, K., Jain, S.K., 2004. Effect of vitamin B-6 on oxygen radicals, mitochondrial membrane potential, and lipid peroxidation in H_2O_2-treated U937 monocytes. Free Radic. Biol. Med. 36, 423–428.

Keles, M., Al, B., Gumustekin, K., Demircan, B., Ozbey, I., Akyuz, M., Yilmaz, A., Demir, E., Uyanik, A., Ziypak, T., Taysi, S., 2010. Antioxidative status and lipid peroxidation in kidney tissue of rats fed with vitamin B-6-deficient diet. Ren. Fail. 32, 618–622.

Kim, H.M., Nguyen Phuoc, L., Min, J.E., Nguyen Hoang, A., Kim, S.J., Yoon, S.J., Kwon, S.W., 2020. Comprehensive phenotyping and multi-omic profiling in the toxicity assessment of nanopolystyrene with different surface properties. J. Hazard. Mater. 399, 123005.

Kolandhasamy, P., Su, L., Li, J., Qu, X., Jabeen, K., Shi, H., 2018. Adherence of microplastics to soft tissue of mussels: a novel way to uptake microplastics beyond ingestion. Sci. Total Environ. 610, 635–640.

Lam, T.W.L., Fok, L., Lin, L., Xie, Q., Li, H.-X., Xu, X.-R., Yeung, L.C., 2020. Spatial variation of floatable plastic debris and microplastics in the Pearl River Estuary, South China. Mar. Pollut. Bull. 158, 111383.

Lanctot, C.M., Bednarz, V.N., Melvin, S., Jacob, H., Oberhaensli, F., Swarzenski, P.W., Ferrier-Pages, C., Carroll, A.R., Metian, M., 2020. Physiological stress response of the scleractinian coral Stylophora pistillata exposed to polyethylene microplastics. Environ. Pollut. 263, 114559.

Law, K.L., Thompson, R.C., 2014. Microplastics in the seas. Science 345, 144–145.

Li, J., Yang, D., Li, L., Jabeen, K., Shi, H., 2015. Microplastics in commercial bivalves from China. Environ. Pollut. 207, 190–195.

Li, J.N., Qu, X.Y., Su, L., Zhang, W.W., Yang, D.Q., Kolandhasamy, P., Li, D.J., Shi, H.H., 2016. Microplastics in mussels along the coastal waters of China. Environ. Pollut. 214, 177–184.

Li, L., Li, R., Zhou, J., Zuniga, A., Stanislaus, A.E., Wu, Y., Huan, T., Zheng, J., Shi, Y., Wishart, D.S., Lin, G., 2013. MyCompoundID: using an evidence-based metabolome library for metabolite identification. Anal. Chem. 85, 3401–3408.

Lu, Y., Zhang, Y., Deng, Y., Jiang, W., Zhao, Y., Geng, J., Ding, L., Ren, H., 2016. Uptake and accumulation of polystyrene microplastics in zebrafish (*Danio rerio*) and toxic effects in liver. Environ. Sci. Technol. 50, 4054–4060.

Magni, S., Gagne, F., Andre, C., Della Torre, C., Auclair, J., Hanana, H., Parenti, C.C., Bonasoro, F., Binelli, A., 2018. Evaluation of uptake and chronic toxicity of virgin polystyrene microbeads in freshwater zebra mussel *Dreissena polymorpha* (Mollusca: Bivalvia). Sci. Total Environ. 631–632, 778–788.

Mazorra, M.T., Rubio, J.A., Blasco, J., 2002. Acid and alkaline phosphatase activities in the clam Scrobicularia plana: kinetic characteristics and effects of heavy metals. Comp. Biochem. Physiol. Part B Biochem. Mol. Biol. 131, 241–249.

Moore, C.J., 2008. Synthetic polymers in the marine environment: a rapidly increasing, long-term threat. Environ. Res. 108, 131–139.

Qiao, R., Sheng, C., Lu, Y., Zhang, Y., Ren, H., Lemos, B., 2019. Microplastics induce intestinal inflammation, oxidative stress, and disorders of metabolome and microbiome in zebrafish. Sci. Total Environ. 662, 246–253.

Sgaravatti, A.M., Vargas, B.A., Zandona, B.R., Deckmann, K.B., Rockenbach, F.J., Moraes, T.B., Monserrat, J.M., Sgarbi, M.B., Pederzolli, C.D., Wyse, A.T.S., Wannmacher, C.M.D., Wajner, M., Dutra-Filho, C.S., 2008. Tyrosine promotes oxidative stress in cerebral cortex of young rats. Int. J. Dev. Neurosci. 26, 551–559.

Song, L., Wu, L., Ni, D., Chang, Y., Xu, W., Xing, K., 2006. The cDNA cloning and mRNA expression of heat shock protein 70 gene in the haemocytes of bay scallop (*Argopecten irradians*, Lamarck 1819) responding to bacteria challenge and naphthalin stress. Fish Shellfish Immunol. 21, 335–345.

Sussarellu, R., Suquet, M., Thomas, Y., Lambert, C., Fabioux, C., Pernet, M.E.J., Le Goïc, N., Quillien, V., Mingant, C., Epelboin, Y., Corporeau, C., Guyomarch, J., Robbens, J., Paul-Pont, I., Soudant, P., Huvet, A., 2016. Oyster reproduction is affected by exposure to polystyrene microplastics. Proc. Natl. Acad. Sci. USA 113, 2430.

Teng, J., Zhao, J., Zhu, X., Shan, E., Zhang, C., Zhang, W., Wang, Q., 2021. Toxic effects of exposure to microplastics with environmentally relevant shapes and concentrations: accumulation, energy metabolism and tissue damage in oyster *Crassostrea gigas*. Environ. Pollut. 269, 116169.

Thushari, G.G.N., Senevirathna, J.D.M., 2020. Plastic pollution in the marine environment. Heliyon 6, 04709.

Voelker, C., Kramm, J., Kerber, H., Schramm, E., Winker, M., Zimmermann, M., 2017. More than a potential hazard-approaching risks from a social-ecological perspective. Sustainability 9, 1039.

Wang, T., Hu, M., Song, L., Yu, J., Liu, R., Wang, S., Wang, Z., Sokolova, I.M., Huang, W., Wang, Y., 2020a. Coastal zone use influences the spatial distribution of microplastics in Hangzhou Bay, China. Environ. Pollut. 266, 115137.

Wang, X., Huang, W., Wei, S., Shang, Y., Gu, H., Wu, F., Lan, Z., Hu, M., Shi, H., Wang, Y., 2020b. Microplastics impair digestive performance but show little effects on antioxidant activity in mussels under low pH conditions. Environ. Pollut. 258, 113691.

Wright, S.L., Thompson, R.C., Galloway, T.S., 2013. The physical impacts of microplastics on marine organisms: a review. Environ. Pollut. 178, 483–492.

Wu, F., Wang, Y., Leung, J.Y.S., Huang, W., Zeng, J., Tang, Y., Chen, J., Shi, A., Yu, X., Xu, X., Zhang, H., Cao, L., 2020. Accumulation of microplastics in typical commercial aquatic species: a case study at a productive aquaculture site in China. Sci. Total Environ. 708, 135432.

Yin, L., Chen, B., Xia, B., Shi, X., Qu, K., 2018. Polystyrene microplastics alter the behavior, energy reserve and nutritional composition of marine jacopever (*Sebastes schlegelii*). J. Hazard. Mater. 360, 97–105.

Zhao, S., Li, L., 2020. Chemical derivatization in LC-MS-based metabolomics study. TrAC Trends Anal. Chem. 131, 115988.

Zhao, S., Zhu, L., Wang, T., Li, D., 2014. Suspended microplastics in the surface water of the Yangtze Estuary System, China: first observations on occurrence, distribution. Mar. Pollut. Bull. 86, 562–568.

Zhao, S., Li, H., Han, W., Chan, W., Li, L., 2019. Metabolomic coverage of chemical-group-submetabolome analysis: group classification and four-channel chemical isotope labeling LC-MS. Anal. Chem. 91, 12108–12115.

Zhu, Z.-l, Wang, S.-c, Zhao, F.-f, Wang, S.-g, Liu, F.-f, Liu, G.-z, 2019. Joint toxicity of microplastics with triclosan to marine microalgae *Skeletonema costatum*. Environ. Pollut. 246, 509–517.