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Polystyrene microplastics induce microbial dysbiosis and dysfunction in surrounding seawater

Guozhu Ye [a,b,1], Xu Zhang [b,c,1], Changzhou Yan [b], Yi Lin [d,*], Qiansheng Huang [a,b,*]

[a] Center for Excellence in Regional Atmospheric Environment, Institute of Urban Environment, Chinese Academy of Sciences, 1799 Jimei Road, Xiamen 361021, China
[b] Key Laboratory of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, 1799 Jimei Road, Xiamen 361021, China
[c] University of Chinese Academy of Sciences, 19 Yuquan Road, Beijing 100049, China
[d] State Key Laboratory of Molecular Vaccinology and Molecular Diagnostics, School of Public Health, Xiamen University, Xiamen 361102, China

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ABSTRACT

Microplastics are ubiquitously present in the environment, accumulate in aquaculture water, and cause toxicological effects on aquatic organisms. Besides, microplastics provide ecological niches for microorganisms in aquatic environments. However, the effects of microplastics on microbial balance and function in surrounding water are still unclear, especially for aquaculture water. Therefore, 16S rRNA gene sequencing was employed to uncover polystyrene microplastics (PS)-induced microbial dysbiosis in surrounding seawater cultivating marine medaka (*Oryzias melastigmas*) and to screen related potential bacterial biomarkers. We found that *Proteobacteria* and *Bacteroidetes* were the dominant phyla in each group, accounting for more than 95% of the total abundance, and that 26 bacterial taxa belonging to *Proteobacteria* and *Bacteroidetes* were significantly altered in surrounding seawater after 10- and 200-μm PS exposure. Functional analysis revelated that photosynthesis, carbon metabolism (such as carbon fixation, glycolysis, tricarboxylic acid cycle, and glycan biosynthesis and metabolism), amino acid metabolism, lipid synthesis, and nucleotide metabolism were decreased, while environmental stress responses, such as xenobiotics biodegradation and metabolism, glutathione metabolism, and taurine and hypotaurine metabolism, were increased in surrounding seawater microbiota after separate 10- and 200-μm PS exposure. Pathway analysis and correlation networks demonstrated that changes in relative abundances of bacterial taxa belonging to *Proteobacteria* and *Bacteroidetes* were highly correlated with those in the liver metabolism of marine medaka. Subsequently, 8 bacterial taxa were discovered to be able to be used separately as the potential biomarker for assessing the surrounding seawater microbial dysbiosis and metabolic responses of marine medaka, with a diagnostic accuracy of 100.0%. This study provides novel insights into toxicological effects of microplastics on microbial dysbiosis and function in surrounding water and ecosystems, and suggests potential roles of biomarkers involved in surrounding microbial dysbiosis in assessing microplastic ecotoxicology, microbial dysbiosis, and the health status of organisms at higher trophic levels.

1. Introduction

Microplastics, as plastic particles with diameters between 1 μm and 5 mm, are ubiquitously present in various waters (such as seawater, coastal waters, and inland freshwaters), soil, sediments, aquatic organisms, foods, and even the human body entering via the drinking water and/or food chain (Brandon et al., 2019; Luo et al., 2019; Toussaint et al., 2019). Microplastics are so small that they can be easily ingested by a variety of organisms, thereby inducing various toxic effects, such as decreases in survival, growth, development and reproduction, hepatic damages, endocrine disorders, neurotoxicity, genotoxicity, oxidative stress, and disordered immunological responses (Gardon et al., 2018; Luo et al., 2019; Pannetier et al., 2020). Accordingly, researches into the ecological effects of microplastics in aquatic ecosystems are of great

Abbreviations: PS, Polystyrene microplastics; PS-10, 10-μm PS; PS-200, 200-μm PS; OUT, operational taxonomic unit; ANOSIM, analysis of similarities; PICRUSt, phylogenetic investigation of communities by reconstruction of unobserved states.

* Corresponding authors at: Center for Excellence in Regional Atmospheric Environment, Institute of Urban Environment, Chinese Academy of Sciences, 1799 Jimei Road, Xiamen 361021, China (Qiansheng Huang).
E-mail addresses: tjlinyi@xmu.edu.cn (Y. Lin), qshuang@iue.ac.cn (Q. Huang).
[1] These authors contributed equally to this work.

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significance.

Accumulated data demonstrates that microbial dysbiosis is closely associated with the toxicological effects induced by environmental pollutants (Feng et al., 2020; Huang et al., 2020; Kakade et al., 2020; Xia et al., 2020). It was reported that dietary exposure to bisphenol A at 50 mg/kg body weight/day for 24 weeks induced accumulation of triglycerides, total cholesterol in the serum and liver, decreases in intestinal tight junction proteins, and increases in markers of hepatic inflammation, such as toll-like receptor 4, phosphorylation of nuclear factor-kappa B, interleukin-1b, and interleukin-6, in male CD-1 mice, and that the diversity of gut microbiota was decreased, and the composition of gut microbiota was changed, which was characterized by an increase in the abundance of *Proteobacteria* (a marker of microbial dysbiosis) and a decrease in the abundance of *Akkermansia*, a gut bacteria correlated with improved gut barrier function and inflammation (Feng et al., 2020). In addition, exposure to Cd at 5 μg/L reduced the survival rate and locomotor activity of zebrafish (Xia et al., 2020). Subsequent high-throughput sequencing analysis showed that the abundance of *Proteobacteria* was increased, while that of *Firmicutes* was decreased after exposure to Cd at 5 μg/L, and that abundances of some bacteria involved in neurodegenerative diseases, such as *Ruminococcaceae*, *Pseudomonas*, *Blautia*, *Bacteroides and Lactobacillus*, were also altered in response to Cd exposure (Xia et al., 2020). Moreover, exposure to polystyrene microplastics (PS) for 28 days revealed that PS could accumulate in the guppy gut, enlarge goblet cells, decrease intestinal activities of digestive enzymes, but increase those of immune cytokines, and that the diversity and evenness of gut microbiota were decreased, and the composition and function of gut microbiota were altered, such as the increase in the abundance of *Proteobacteria*, and the suppression of metabolism and repair pathway (Huang et al., 2020).

An investigation of microplastic abundance and characteristics in aquaculture water and influents from the Pearl River Estuary (Guangzhou, China) discovered that the average concentrations of both total and small-sized (<100 μm) microplastics in aquaculture water were higher than those in pond influents, indicating that microplastics from water in the Pearl River Estuary could accumulate in aquaculture water (Ma et al., 2020). A study of microplastic contamination in three coastal regions found that microplastic abundance was higher in sediments from the aquafarm and urban site than that from the rural site, and that PS abundance was higher in the aquafarm site than that in the urban and rural site (Jang et al., 2020). Besides, microplastics can work as stable substrates and ecological niches for microorganisms in aquatic environments (Wen et al., 2020; Xue et al., 2020; Yang et al., 2020). It was revealed that the diversity and evenness of bacteria on microplastics were higher than those in surrounding water, and that metabolic pathways in the community on microplastics were generally enriched, such as metabolism and transport of carbohydrates, amino acids, and inorganic ions (Wen et al., 2020; Xue et al., 2020). However, the effects of microplastics on the microecology of surrounding water are still unclear, especially for aquaculture water. Therefore, 10- and 200-μm PS (PS-10 and PS-200) were firstly separately added to the culture water of marine medaka (*Oryzias melastigmas*) daily for a continuous 60-day exposure to induce water microbial dysbiosis. Subsequently, the surrounding seawater was collected for 16S rRNA gene sequencing to characterize the microbial dysbiosis induced by PS, and to discover relevant potential bacterial markers.

2. Materials and methods

2.1. Materials

PS-10 and PS-200 were ordered from Shanghai Guanbu Electromechanical Technology Co., Ltd (Shanghai, China). The salt was the product of China Salt Engineering Technology Research Institute Co., Ltd. (China). Polycarbonate membranes with 0.22-μm pore sizes were obtained from Millipore Corp. (USA). The FastDNA® Spin Kit for Soil was purchased from MP Biomedicals (USA).

2.2. PS exposure experiments

The salinity of the artificial seawater was 30‰, with KH of 7–10, and pH of 8.0–8.4. Adult marine medaka (male/femal, 1:1) aged 8 months were cultured in the seawater, which was successively filtered by 3 layers of filter papers and 0.22-μm filter membranes to remove potential microbes. It is revealed that the number concentration range of microplastics in drinking water and freshwater is from 1×10^{-2} to 10^{8} particles/m^3, and that microplastic exposure at a concentration from 2 μg/L to 40 mg/L can trigger various toxic effects, e.g., microplastic accumulation in the intestine, microbiota dysbiosis, inflammation, and metabolic disturbances (Foekema et al., 2013; Koelmans et al., 2019; Lu et al., 2016; Qiao et al., 2019; Wang et al., 2019; Yu et al., 2018). Accordingly, marine medaka was separately exposed to 10 mg/L of PS-10 and PS-200 in the seawater, corresponding to 1.82×10^{10} and 2.27×10^{6} particles/m^3 for PS-10 and PS-200, respectively. Besides, the seawater was continuously aerated to assure the even distribution of microplastics for the free intake. Moreover, the seawater was renewed daily during the 60-day exposure period.

2.3. Sample preparation for 16S rRNA gene sequencing

A 0.22-μm polycarbonate membrane was used to filter 500-mL seawater from each group, and then placed in a sterilized centrifuge tube and stored at −80 °C. The filter membrane was cut into pieces and placed in a new centrifuge tube. The microbial DNA on the filter membrane was extracted using the FastDNA® Spin Kit for Soil according to the instructions, and the purity and concentration were detected by agarose gel electrophoresis.

2.4. 16S rRNA gene sequencing

After dilution to 1 ng/μL with sterile water, the genomic DNA was used as the template. The specific primer set (515F: 5́-GTGCCAGCMGCCGCGGTAA-3́; 806R:5́-GGACTACHVGGGTWTCTAAT-3́) with the barcode was used to amplify the V4 region of 16S rRNA gene. PCR products were detected by electrophoresis with 2% agarose gel and mixed in equidensity ratios according to the concentration. After full mixing, the mixed products were purified using Qiagen Gel Extraction Kit (Qiagen, Germany) to obtain the target bands. TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) was used to generate sequencing libraries following manufacturer's instructions. Purified libraries were evaluated by the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. Ultimately, the library was sequenced using an Illumina HiSeq2500 PE250 platform, and 250 bp paired-end reads were produced.

2.5. Data preprocessing

After assignment to samples according to the unique barcode, paired-end reads were truncated by deleting the primer sequence and barcode, and then merged employing FLASH 1.2.7 (Magoč and Salzberg, 2011). The splicing sequences were defined as raw tags, which were filtered to generate high-quality clean tags based on the quality-controlled process of QIIME 1.7.0 (Caporaso et al., 2010). Following comparison with the Gold database using the UCHIME algorithm, the chimeric sequences were detected and then removed to obtain the effective tags (Edgar et al., 2011). Sequences with similarities of not <97% were classified as the same operational taxonomic unit (OTU). The representative sequence of each OTU was annotated to the corresponding taxonomic information according to the GreenGene Database using the Ribosomal Database Project Classifier algorithm (DeSantis et al., 2006; Wang et al., 2007). The abundance of OTU was normalized based on the sequence number of the sample with the least sequences. After that, the data was

defined as the relative abundance, and used for subsequent statistical analysis.

2.6. Statistical analysis

MeV 4.9.0 was employed for plotting the heat map (Saeed et al., 2006). Principal co-ordinates analysis, principal component analysis, ANOSIM (analysis of similarities) tests, Cladograms, linear discriminant analysis, and PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states) analysis were conducted via the Novomagic platform (https://magic.novogene.com). The two-tailed Mann-Whitney *U* test performed by PASW Statistics 18 (SPSS Inc., Chicago, USA) was applied to compare differences in the relative abundance and function of microbiota between the control and PS exposure group. Spearman correlations between bacterial taxa and metabolites were performed by PASW Statistics 18. Correlation networks of bacteria taxa and metabolites were constructed with Cytoscape 2.8.2 (Smoot et al., 2010). A binary logistic regression model was constructed to assess the bacterial taxa as potential biomarkers for distinguishing PS-induced seawater microbial dysbiosis and metabolic responses of marine medaka liver using PASW Statistics 18. The receiver operating characteristic curve was used to evaluate the diagnostic performance of the binary logistic regression employing PASW Statistics 18. The statistically significant level was 0.05.

3. Results

3.1. PS exposure induces significant changes in seawater microbial community composition

Data on the quality control of 16S rRNA data showed that the percentage of bases with a base quality value greater than 20 and 30 (corresponding to the sequencing error rate <1% and 0.1%, respectively) in the effective tags in each sample were bigger than 99% and 98%, respectively, and that the percentage of the number of effective tags to the number of raw paired-end reads in each sample was bigger than 93%, demonstrating the high extraction efficiency and reliability of the sequencing data in this study (Table S1). Therefore, 16S rRNA gene sequencing data were used for subsequent statistical analysis.

Principal co-ordinates analysis and ANOSIM tests revealed that the seawater microbial community structure of both PS-10 and PS-200 groups differed greatly from that of the control group (Fig. 1A and B). *Proteobacteria* and *Bacteroidetes*, the dominant phyla in each group, accounts for more than 95% of the total relative abundance (Fig. 1C). Subsequent linear discriminant effect size analysis showed that bacteria were enriched in the taxa belonging to *Proteobacteria* phylum in PS-10 and PS-200 groups, while bacteria belonging to *Bacteroidetes* phylum predominated in the control group (Fig. 1D and E).

Further comparisons revealed that relative abundances of 27

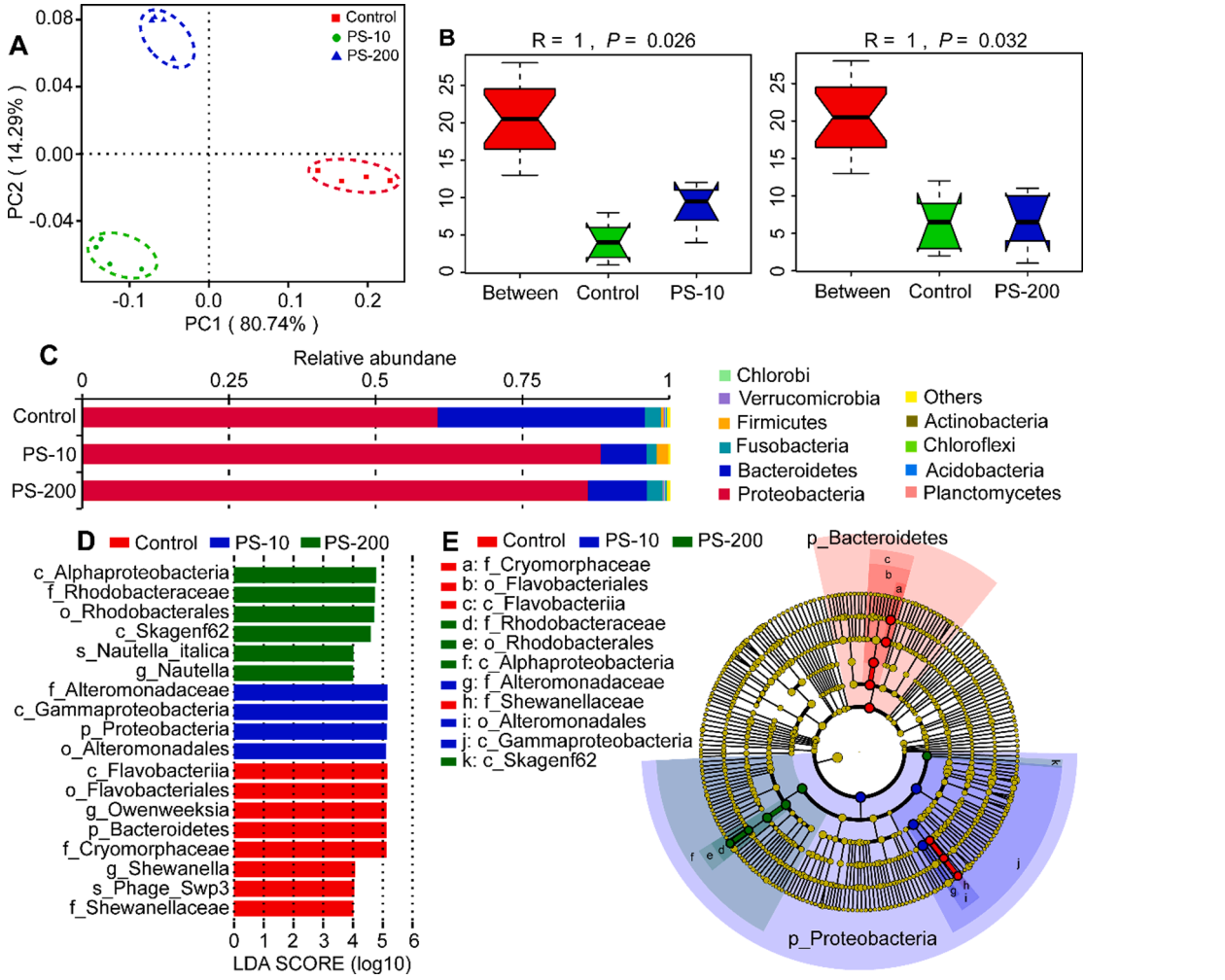

Fig. 1. PS exposure induces significant changes in seawater microbial community composition. n = 4 per group. (A) Principal co-ordinates analysis of PS-induced changes in microbial community composition. (B) ANOSIM tests on microbial community structure differences between groups. (C) The microbial composition at the phylum level in each group. (D) Linear discriminant analysis (LDA) score of the predominant bacteria in each group. (E) Cladograms showing the predominant bacteria in each group.

bacterial taxa were significantly altered after PS exposure, including 2, 2, 3, 10, 8 and 2 bacterial taxa at the phylum, class, order, family, genus, and species level, respectively (Fig. 2). Specifically, relative abundances of the *Proteobacteria* phylum and its subordinate class *Gammaproteobacteria*, order *Alteromonadales*, families *Alteromonadaceae*, *Halieaceae*, *Nannocystaceae*, *Pseudomonadaceae* and *Phyllobacteriaceae*, and genera *Bdellovibrio* and *Pseudomonas* were significantly increased, while relative abundances of order *Sphingomonadales*, families *Erythrobacteraceae* and *Idiomarinaceae*, genera *Erythrobacter* and *Idiomarina*, and species *Erythrobacter citreus*, belonging to the *Proteobacteria* phylum, were significantly decreased in response to PS exposure. Additionally, relative abundances of the *Bacteroidetes* phylum and its subordinate class *Flavobacteriia*, order *Flavobacteriales*, families *Cryomorphaceae*, *Marinilabiaceae* and *Rhodothermaceae*, and genera *Owenweeksia* and *Carboxylicivirga* were significantly decreased, while the genus *Fulvivirga* and species *Marivirga tractuosa* were significantly increased after PS exposure.

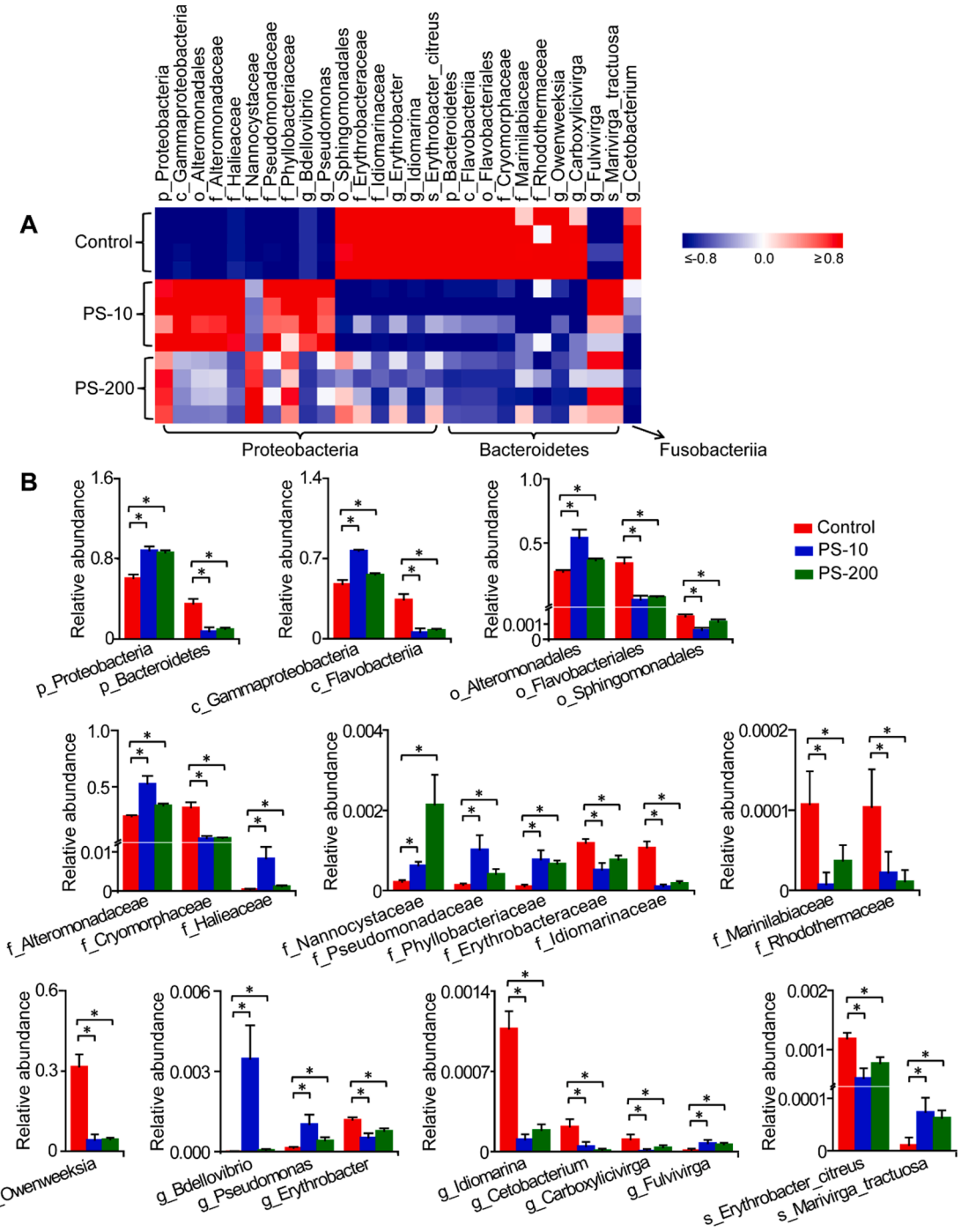

Fig. 2. PS exposure induces significant changes in the relative abundance of seawater microbiota at different taxa levels. The changes were presented by heat map (A) and column (B) plot, respectively. Only the bacteria whose relative abundances significantly altered with the same trend upon separate PS-10 and PS-200 exposure are displayed. After relative abundances of bacterial taxa were normalized to unit variance, the data were used for heat map plot. The column is expressed as the mean + SD. n = 4 per group. $^*P < 0.05$, two-tailed Mann-Whitney *U* test.

3.2. PS exposure induces significant changes in predicted seawater microbial function

A PICRUSt analysis was employed to predict significant changes in the function of microbial community. Principal component analysis showed that the function of seawater microbes was significantly altered upon PS exposure (Fig. 3A). The pathway annotation revealed that 49.76% of total enriched genes were enriched in pathways related to metabolism, followed by pathways related to genetic information processing, unclassified pathways, and pathways related to environmental information processing, separately accounting for 16.01%, 15.27% and 12.37% of total enriched genes, respectively (Fig. 3B). Moreover, amino acid metabolism, membrane transport, carbohydrate metabolism, replication and repair, and energy metabolism were the main known

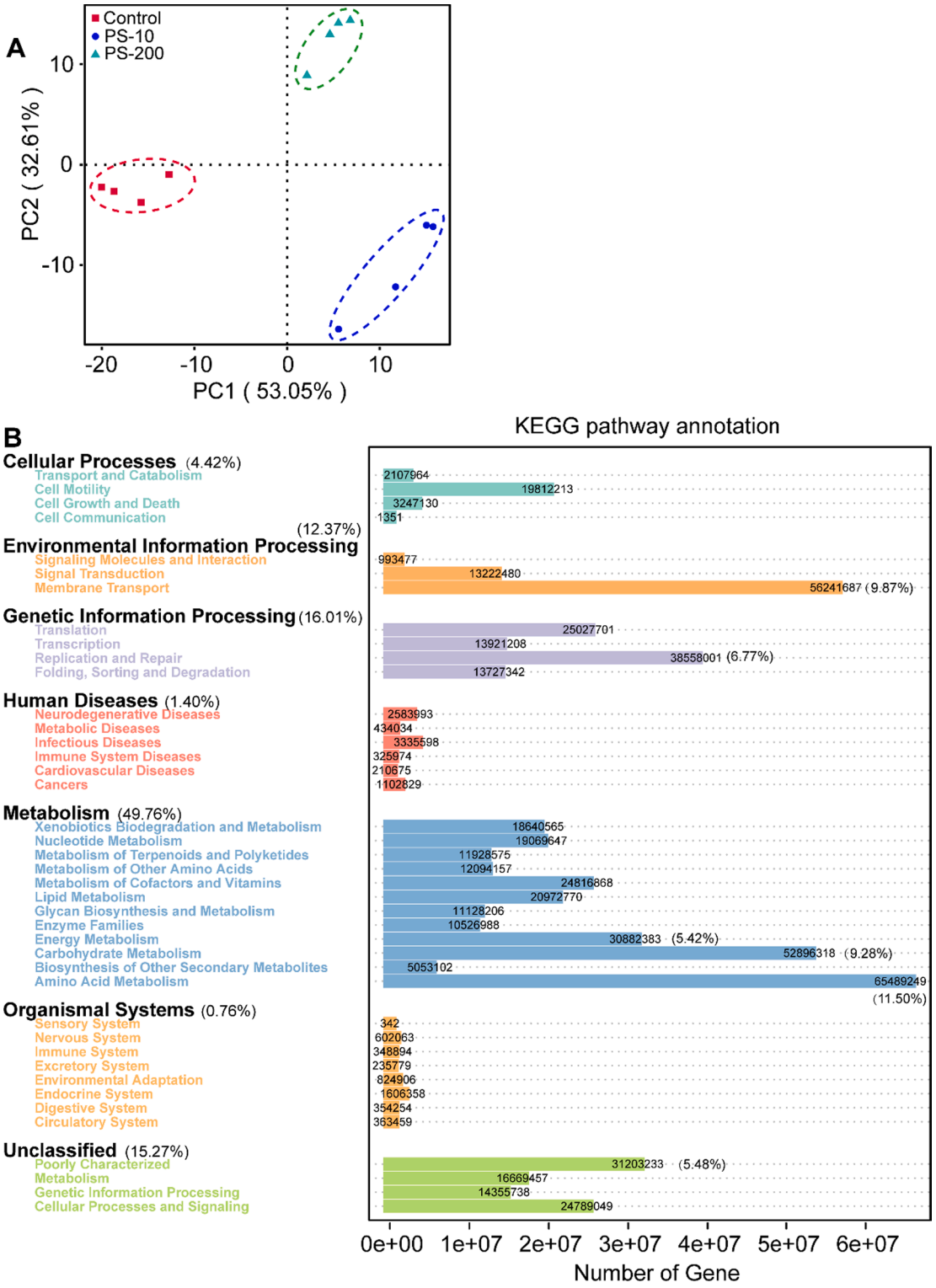

Fig. 3. PS exposure induces significant changes in predicted seawater microbiota function profiles. (A) Sample distributions in the score plot of principal component analysis. (B) Results of the pathway annotation. n = 4 per group.

pathways with the highest enrichment, ratio of total enriched genes in each pathway all above 5.0% (Fig. 3B). Totally, 211 and 230 pathways were predicted to be significantly altered in seawater microbes responding to PS-10 and PS-200 exposure, respectively, of which 167 pathways were in intersection (Tables S2 and S3). Owing to the highest enrichment of pathways related to metabolism in seawater microbes exposed to PS, changes in metabolic pathways in seawater microbes were further explored.

3.3. PS exposure induces significant changes in amino acid metabolism in seawater microbiota

The function prediction showed that amino acid related enzymes, histidine metabolism, lysine biosynthesis, phenylalanine, tyrosine and tryptophan biosynthesis, tyrosine metabolism, and D-glutamine and D-glutamate metabolism were significantly inhibited, while lysine degradation, and cyanoamino acid metabolism were significantly activated, which implied the inhibition of amino acid metabolism, and accelerated degradation of amino acids and proteins in PS-exposed seawater microbiota (Fig. 4). Additionally, metabolic pathways related to redox were also significantly altered in PS-exposed seawater microbiota (Fig. 4). Glutathione metabolism, and taurine and hypotaurine metabolism were significantly activated, whereas selenocompound metabolism was significantly suppressed, which suggested the existence of oxidative stress and the activation of the antioxidant system in PS-exposed seawater microbiota.

3.4. PS exposure induces significant changes in nucleotide, carbohydrate, and lipid metabolism in seawater microbiota

Purine metabolism and pyrimidine metabolism were significantly suppressed, suggesting disordered energy production and/or nucleic acid synthesis in PS-exposed seawater microbiota (Fig. 4). In addition, carbohydrate metabolism was significantly altered in PS-exposed seawater microbiota (Fig. 4). Ascorbate and aldarate metabolism, tricarboxylic acid cycle, glycolysis/gluconeogenesis, pentose phosphate pathway, and pyruvate metabolism were significantly suppressed, while short-chain organic acid metabolism (butanoate metabolism, and propanoate metabolism) was significantly activated, which suggested a decrease in carbohydrate metabolism and disordered energy production in PS-exposed seawater microbiota. Moreover, lipid metabolism was significantly altered in PS-exposed seawater microbiota (Fig. 4). Arachidonic acid metabolism, biosynthesis of unsaturated fatty acids, and fatty acid metabolism were significantly activated, while fatty acid biosynthesis, glycerolipid metabolism, linoleic acid metabolism, and lipid biosynthesis proteins were significantly suppressed, which implied decreases in fatty acid biosynthesis and glycerolipid metabolism, and an increase in fatty acid desaturation, oxidation and/or elongation in PS-

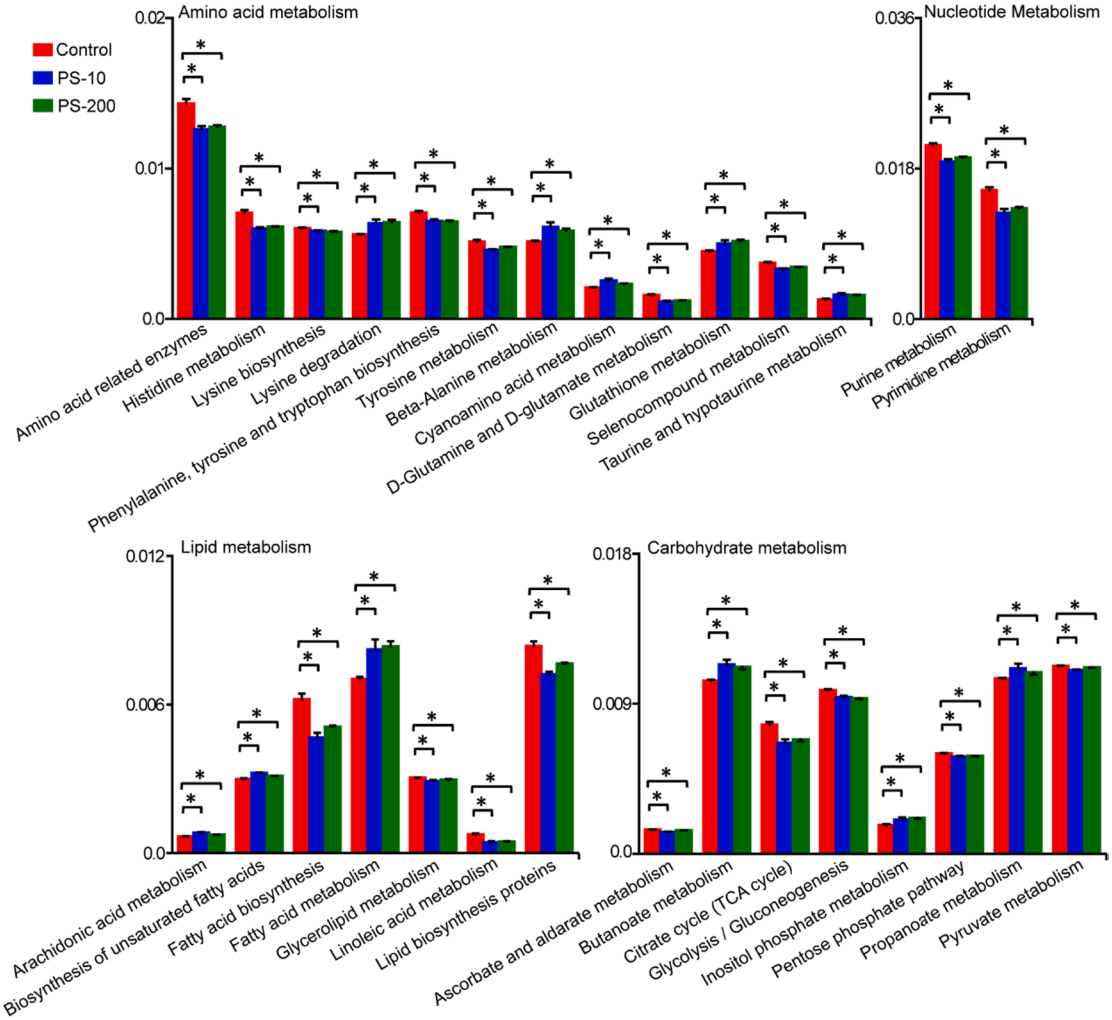

Fig. 4. PS exposure induces significant changes in amino acid, nucleotide, lipid, and carbohydrate metabolism in seawater microbiota. Only the metabolic pathways significantly altered with the same trend responding to separate PS-10 and PS-200 exposure are displayed ($P < 0.05$). The column is expressed as the mean + SD. n = 4 per group. *$P < 0.05$, two-tailed Mann-Whitney U test.

exposed seawater microbiota.

3.5. PS exposure induces significant changes in metabolism of glycans, terpenoids, and polyketides in seawater microbiota

We also observed that 9 predicted metabolic pathways related to glycan biosynthesis and metabolism were significantly suppressed, such as glycosaminoglycan degradation, glycosphingolipid biosynthesis - ganglio series, lipopolysaccharide biosynthesis, N-glycan biosynthesis, and peptidoglycan biosynthesis, which suggested decreases in glycan biosynthesis and degradation in PS-exposed seawater microbiota (Fig. 5). In addition, 9 metabolic pathways related to metabolism of terpenoids and polyketides were significantly suppressed, such as biosynthesis of ansamycins, carotenoid biosynthesis, terpenoid backbone biosynthesis, and tetracycline biosynthesis, whereas biosynthesis of 12-, 14- and 16-membered macrolides, geraniol degradation, and limonene and pinene degradation were activated, which implied an decrease in biosynthesis of terpenoids and polyketides, and an increase in degradation of terpenoids and polyketides in PS-exposed seawater microbiota (Fig. 5).

3.6. PS exposure induces significant changes in other metabolic pathways in seawater microbiota

We found that 5 metabolic pathways related to biosynthesis of other secondary metabolites were significantly suppressed, including flavonoid biosynthesis, Isoquinoline alkaloid biosynthesis, novobiocin biosynthesis, streptomycin biosynthesis, and tropane, piperidine and pyridine alkaloid biosynthesis, whereas 8 metabolic pathways related to xenobiotics biodegradation and metabolism were activated, such as aminobenzoate degradation, caprolactam degradation, dioxin degradation, chloroalkane and chloroalkene degradation, and metabolism of xenobiotics by cytochrome P450, which suggested a decrease in biosynthesis of other secondary metabolites, and accelerated degradation of xenobiotics degradation in PS-exposed seawater microbiota (Fig. 6).

Cofactors, vitamins, and energy were essential for many metabolic pathways and other biological processes, such as amino acid metabolism, tricarboxylic acid cycle, fatty acid metabolism, and methylation modification of genes/proteins. We observed that 6 metabolic pathways related to metabolism of cofactors and vitamins, such as biotin

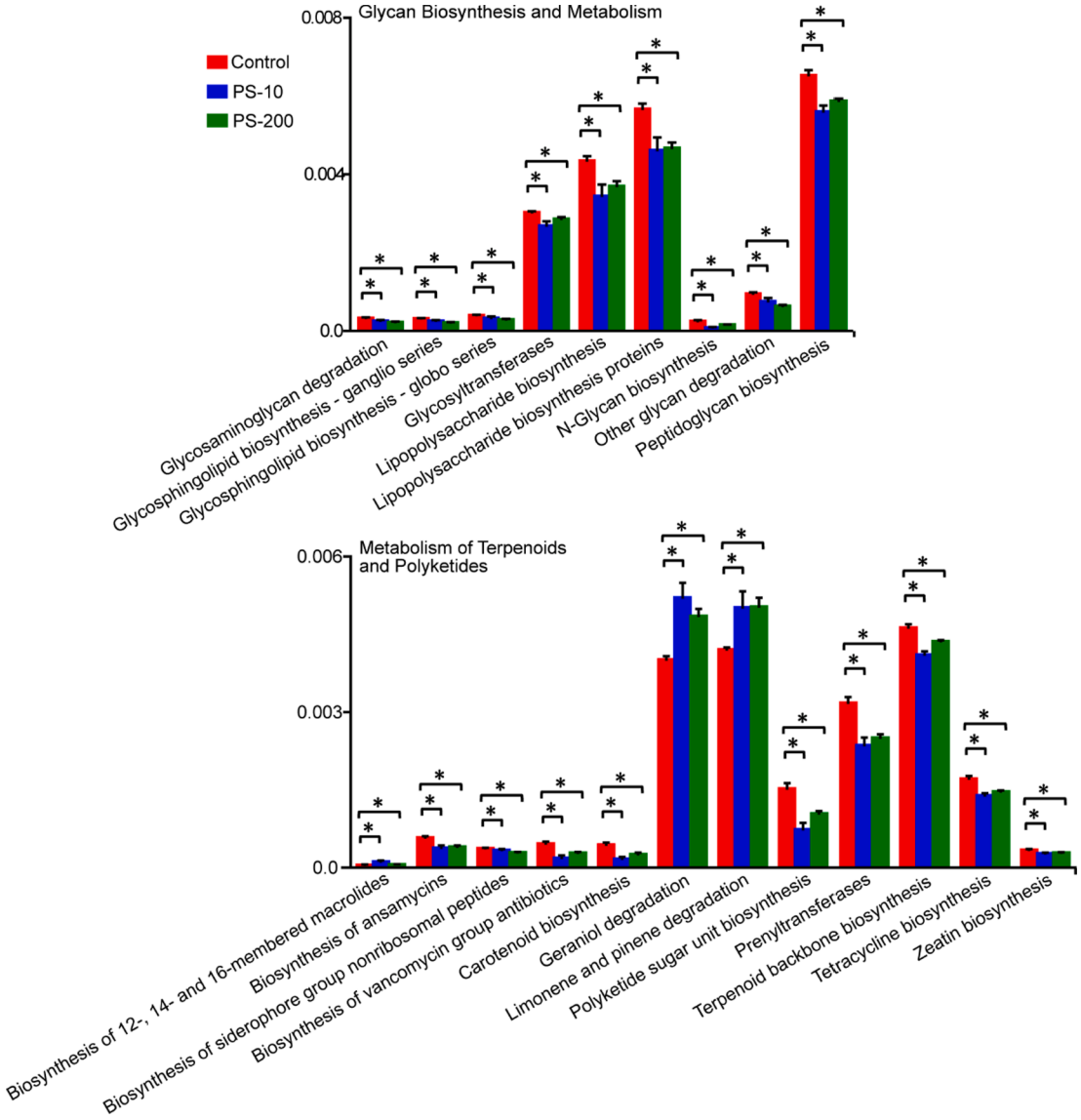

Fig. 5. PS exposure induces significant changes in glycan, terpenoid, and polyketide metabolism in seawater microbiota. Only the pathways significantly altered with the same trend responding to separate PS-10 and PS-200 exposure are displayed ($P < 0.05$). The column is expressed as the mean + SD. n = 4 per group. * $P < 0.05$, two-tailed Mann-Whitney U test.

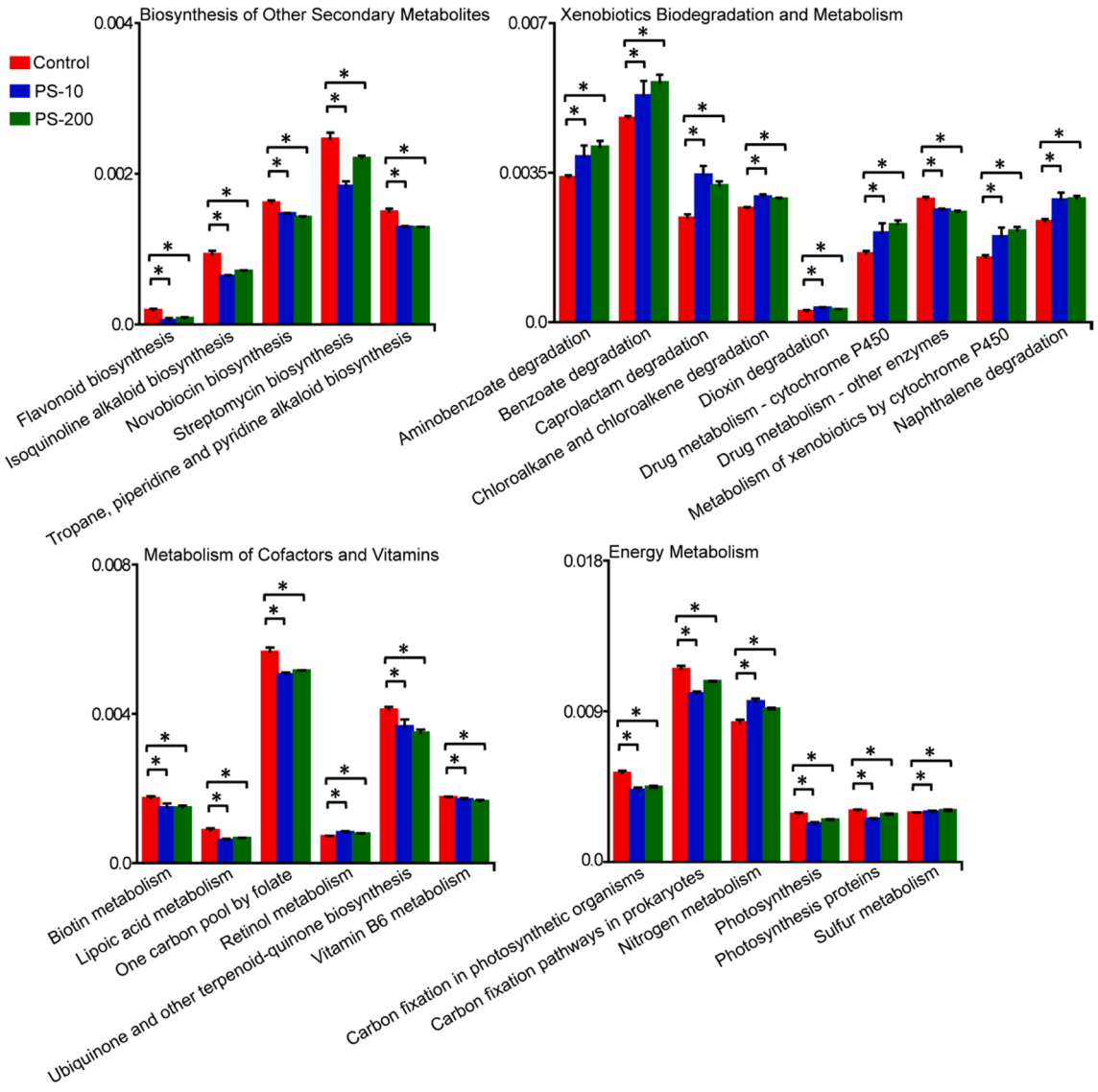

Fig. 6. PS exposure induces significant changes in other metabolic pathways in seawater microbiota. Only the pathways significantly altered with the same trend responding to separate PS-10 and PS-200 exposure are displayed ($P < 0.05$). The column is expressed as the mean + SD. n = 4 per group. *$P < 0.05$, two-tailed Mann-Whitney U test.

metabolism, one carbon pool by folate, and vitamin B6 metabolism, were significantly suppressed in PS-exposed seawater microbiota (Fig. 6). Besides, 4 metabolic pathways related to energy metabolism, including carbon fixation in photosynthetic organisms, carbon fixation pathways in prokaryotes, photosynthesis, and photosynthesis proteins, were significantly suppressed, while sulfur metabolism, and nitrogen metabolism were significantly increased in PS-exposed seawater microbiota (Fig. 6). The suppressed metabolism of cofactors and vitamins, carbon fixation, and photosynthesis were consistent with decreases in metabolism of nucleotides, carbohydrates, glycans, terpenoids and polyketides, and other secondary metabolites, lipid synthesis, and amino acid related enzymes in PS-exposed seawater microbiota. Moreover, the activation of nitrogen metabolism and sulfur metabolism was in accordance with increases in glutathione metabolism, and taurine and hypotaurine metabolism to increase resistance to adverse circumstances in PS-exposed seawater microbiota.

3.7. *High correlations of microbial dysbiosis in surrounding seawater with the liver metabolism of marine medaka*

Among the predicted changes in pathways in seawater microbes, 23 and 20 metabolic pathways were also significantly altered in marine medaka liver in response to PS-10 and PS-200 exposure, respectively, such as biosynthesis of unsaturated fatty acids, glycerolipid metabolism, glycolysis/gluconeogenesis, propanoate metabolism, pentose phosphate pathway, taurine and hypotaurine metabolism and beta-alanine metabolism (Fig. 7, Tables S2 and S3). These data suggested that seawater bacterial indicators could be used as a useful tool to predict and assess metabolic responses of marine medaka liver to PS exposure.

Spearman correlations were used to evaluate the relationship between bacteria taxa and the liver metabolism of marine medaka. It was obvious that levels of many metabolites, including monosaccharides, organic acids, amino acids, methyl and ethyl esters and nucleotides, were significantly correlated with relative abundances of bacteria taxa in response to PS exposure (Fig. S1 and Table S4). To further explore the latent relationships between bacteria taxa and the liver metabolism of marine medaka, absolute values of Spearman correlation coefficients

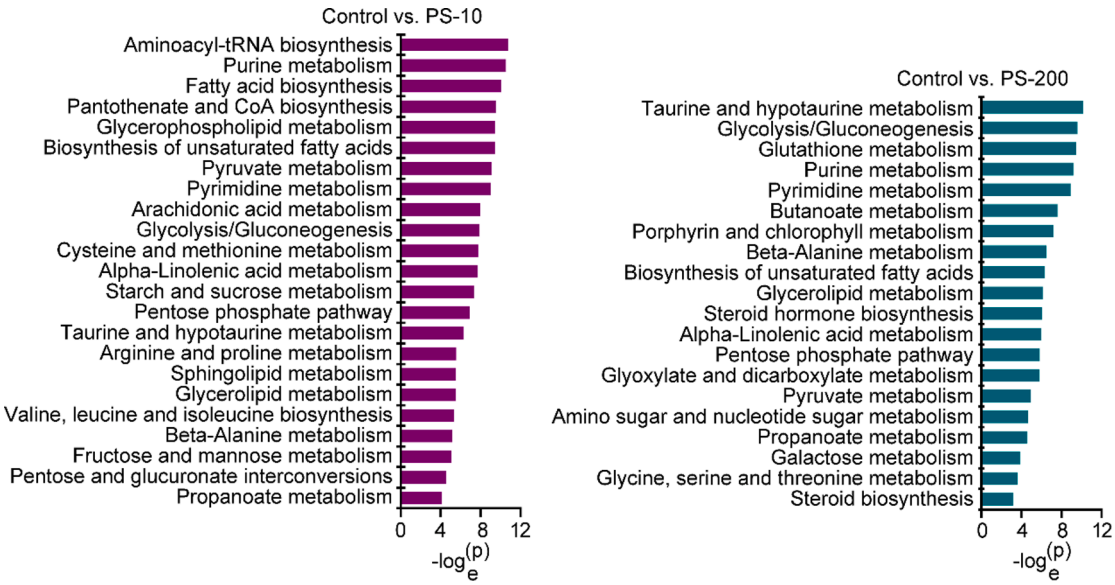

Fig. 7. PS exposure induces common significant changes in metabolic pathways in both the seawater microbiota and marine medaka liver. Only the metabolic pathways significantly altered in both marine medaka liver and seawater bacteria are displayed ($P < 0.05$). n = 4 per group. The metabolomics data are from our study, which is published in Science of the Total Environment (Ye et al., 2021).

were set to be higher than 0.8 to construct correlation networks (Fig. 8). Glucose, mannose and lactate, involved in glycolysis/gluconeogenesis, positively correlated with the *Bacteroidetes* phylum and its subordinate class *Flavobacteriia* and order *Flavobacteriales*, and certain subdivisions of *Proteobacteria* phylum, including order *Sphingomonadales*, families *Erythrobacteraceae* and *Idiomarinaceae*, genera *Erythrobacter* and *Idiomarina*, and species *Erythrobacter citreus*, while negatively correlated with the *Proteobacteria* phylum and its subordinate order *Alteromonadales*, families *Alteromonadaceae* and *Halieaceae* and *Pseudomonadaceae*, and genera *Bdellovibrio* and *Pseudomonas* (Fig. 8A). Strong correlations of metabolites involved in other carbohydrate metabolism, such as pentose phosphate pathway and tricarboxylic acid cycle, with bacteria taxa could be observed as well (Fig. 8A).

High correlations of metabolites involved in amino acid metabolism (including valine, leucine and isoleucine metabolism, beta-alanine metabolism, alanine, asparate and glutamate metabolism, arginine and proline metabolism and taurine and hypotaurine metabolism) and nucleotide metabolism with phyla *Proteobacteria* and *Bacteroidetes* and their subdivisions were found as well (Fig. 8B and C). It was notable that fatty acids (including stearidonate and pentadecanoate), fatty acid methyl and ethyl esters (methyl 7,10,13,16,19-docosapentaenoate, ethyl 9-hexadecenoate, ethyl linoleate, ethyl oleate and ethyl elaidate) positively correlated with the *Proteobacteria* phylum and its subordinate class *Gammaproteobacteria*, order *Alteromonadales*, families *Alteromonadaceae*, *Halieaceae* and *Pseudomonadaceae*, and genera *Bdellovibrio* and *Pseudomonas*, while negatively correlated with some other subdivisions of *Proteobacteria* phylum, including order *Sphingomonadales*, families *Erythrobacteraceae* and *Idiomarinaceae*, genera *Erythrobacter* and *Idiomarina*, and species *Erythrobacter citreus*, and that correlations of fatty acids, fatty acid methyl and ethyl esters with the *Proteobacteria* phylum and its subdivisions were closer than those between the bacterial taxa and other metabolites (Fig. 8D). These data demonstrated that changes in relative abundances of phyla *Proteobacteria* and *Bacteroidetes* and their subdivisions in the seawater were highly correlated with those in carbohydrate metabolism, amino acid metabolism, nucleotide metabolism, and lipid metabolism in marine medaka liver.

3.8. Potential bacterial biomarkers for assessing the seawater microbial dysbiosis and metabolic responses of marine medaka

Owing to high correlations of seawater bacterial taxa with the liver metabolism of marine medaka, significantly altered bacterial taxa with fold changes above 2 in response to PS exposure were further employed as potential bacterial biomarkers to assess the seawater microbial dysbiosis and metabolic responses of marine medaka. Subsequently, 8 bacterial taxa, including families *Halieaceae*, *Nannocystaceae*, *Pseudomonadaceae* and *Phyllobacteriaceae*, genus *Bdellovibrio*, *Pseudomonas* and *Fulvivirga*, and species *Marivirga tractuosa*, were used separately as the potential biomarker (Fig. 9). Binary logistic regression results showed that the above bacteria taxa used separately as the bacterial biomarker were able to correctly distinguish the seawater microbial dysbiosis and metabolic responses of marine medaka, and that the accurate rate was 100% (Fig. 9A). Subsequent receiver operating characteristic curve analysis of the diagnosis by binary logistic regression revealed that the area under the receiver operating characteristic curve was 1.0, and that the sensitivity and specificity were all 100%, demonstrating the excellent diagnostic performance (Fig. 9B). These data proved that the bacteria taxa could be used separately as the potential biomarker for evaluating the surrounding seawater microbial dysbiosis and metabolic responses of marine medaka.

4. Discussion

Due to their widespread presence in the environment and their potential toxicological effects on organisms and humans, microplastics have received great attentions. Accumulated data shows that microbial dysbiosis is closely correlated with the toxicological effects of environmental pollutants, and that microplastics from the river can accumulate in aquaculture water (Feng et al., 2020; Huang et al., 2020; Kakade et al., 2020; Ma et al., 2020; Xia et al., 2020). Moreover, microplastics can provide novel ecological niches for microorganisms in aquatic environments (Wen et al., 2020; Xue et al., 2020; Yang et al., 2020). However, the effects of microplastics on the microecology of surrounding water are undefined, especially for aquaculture water. Accordingly, 16S rRNA gene sequencing was used to characterize PS-induced microbial dysbiosis in the surrounding seawater cultivating marine medaka and to discover relevant potential bacterial biomarkers

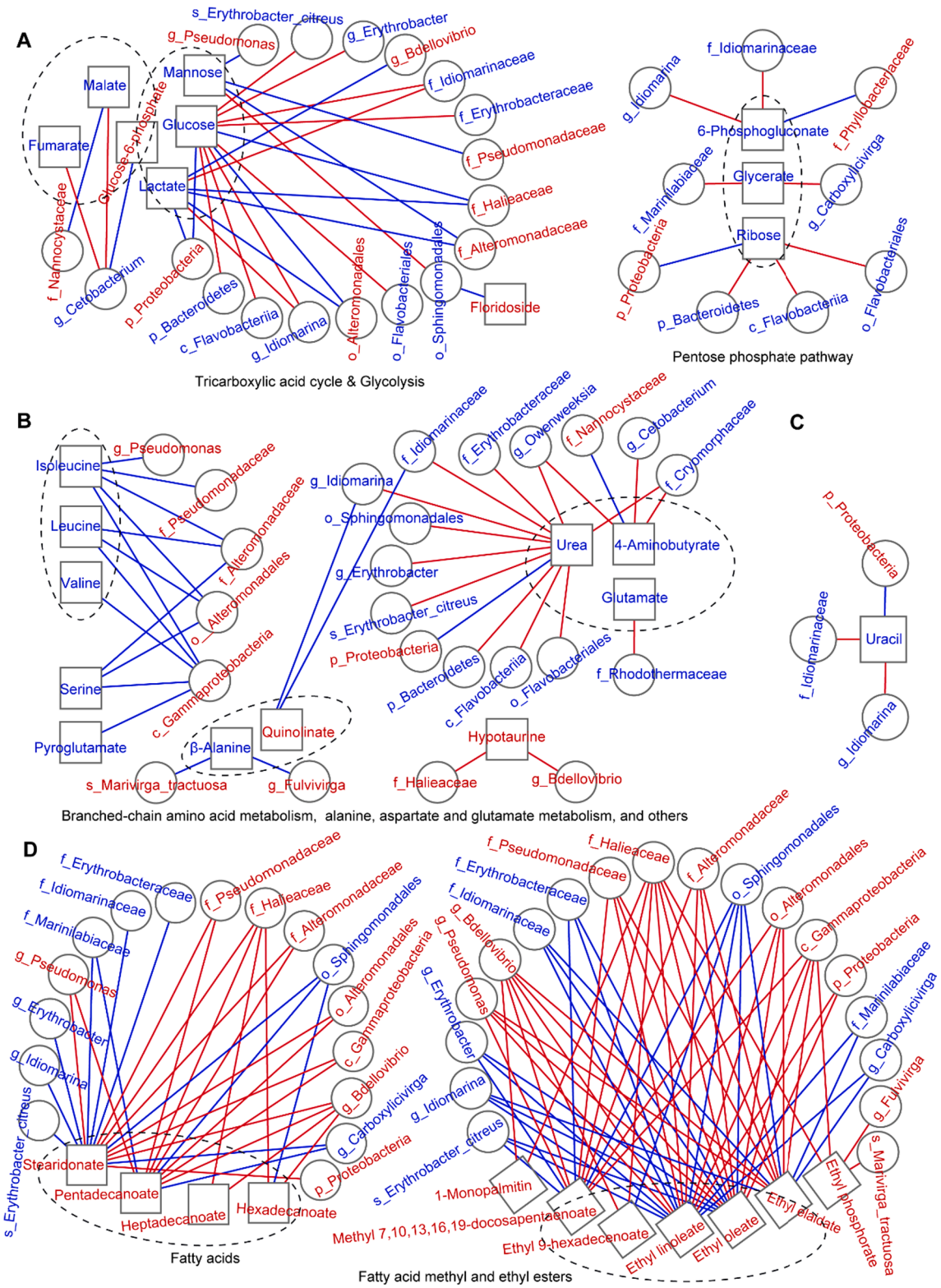

Fig. 8. Correlation networks of bacterial taxa and metabolites in response to PS exposure. Circle, bacterial taxa; square, metabolites. Red/blue lines: positive/negative correlations. Metabolites in red/blue fonts: significantly increased/decreased in PS-10 and/or PS-200 groups compared to the control group. Bacterial taxa in red/blue fonts: significantly increased/decreased in PS-10 and PS-200 groups compared to the control group. Spearman correlations between bacterial taxa and metabolites involved in carbohydrate metabolism (A) amino acid metabolism (B) nucleotide metabolism (C) and lipid metabolism (D) are displayed. Only bacteria whose abundances significantly altered with the same trend responding to separate PS-10 and PS-200 exposures were used for correlation analysis. The absolute value of the correlation coefficient is higher than 0.8. n = 10 per group. The metabolomics data are from our study, which is published in Science of the Total Environment (Ye et al., 2021). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

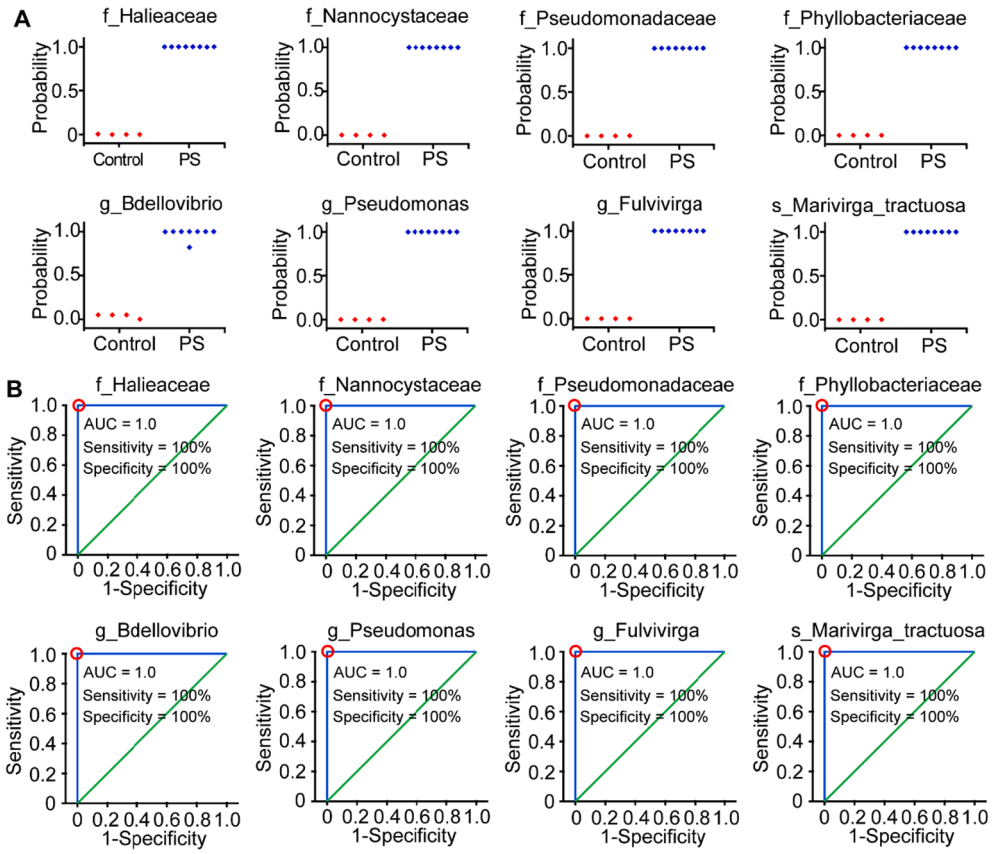

Fig. 9. Potential bacterial biomarkers for assessing the seawater microbial dysbiosis and metabolic responses of marine medaka. (A) Eight bacterial taxa used separately as the potential biomarker for assessing the seawater microbial dysbiosis and metabolic responses of marine medaka. (B) The diagnosis performance. The red circle in the receiver operating characteristic curve indicates the data point with the best diagnostic performance (the highest sensitivity and specificity). AUC, area under the receiver operating characteristic curve. n = 4 and 8 in the control and PS group, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in this study.

We found PS-induced decreases in predicted photosynthesis and carbon fixation, which was in accordance with decreases in many metabolic pathways, such as metabolism of nucleotides, carbohydrates, glycans, terpenoids and polyketides, and other secondary metabolites, in seawater microbiota. The suppression of photosynthesis will probably affect the growth of photosynthetic autotrophic and heterotrophic organisms. Metatranscriptomics revealed that transcripts related to energy production (such as oxidative phosphorylation, photosynthesis, bacteriochlorophyll a, and proteorhodopsins), purine metabolism, and pyrimidine metabolism were enriched in the day compared to the night, and that *Bacteroidetes* was one of the phyla with the highest transcriptional activity in the community (Vila-Costa et al., 2013). *Bacteroidetes* were found to produce energy by establishing H^+ gradients across the membrane via proteorhodopsins in the day and pyrophosphatases at night (Vila-Costa et al., 2013). Accordingly, the suppression of predicted photosynthesis, carbon fixation, and related carbon compound metabolism was probably due to decreased relative abundances of the *Bacteroidetes* phylum in PS-exposed seawater microbiota.

Functional analysis revealed that pathways related to environmental stress responses, such as glutathione metabolism, sulfur metabolism, taurine and hypotaurine metabolism, and xenobiotics biodegradation and metabolism, were activated in PS-exposed seawater microbiota in this study, which indicated the occurrence of environmental stresses and resultant activation of stress responses after PS exposure. It was showed that levels of malondialdehyde, interleukin 1β/6, and tumor necrosis factor alpha were increased, while those of glutathione, glutathione peroxidase, superoxide dismutase, and interleukin 10 were decreased in cisplatin-triggered intestinal mucositis accompanied by high relative abundances of *Proteobacteria* and *Deferribacteres*, and that D-methionine, a sulfur-containing amino acid, could alleviate the above oxidative stress and inflammatory reaction in cisplatin-triggered intestinal mucositis (Wu et al., 2019). High-throughput 16S rRNA gene sequencing and metagenomic functional prediction revealed that *Proteobacteria* was the predominant phylum, followed by *Firmicutes*, *Actinobacteria* and *Bacteroidetes* in soils of a Pb/Zn smelter, and that metal exportation and biotransformation, and antioxidant response play roles in bio-removal of and bio-resistance to heavy metals in soils of a Pb/Zn smelter (Li et al., 2020). In addition, stable isotope probing and 16S rRNA gene sequencing indicated that the phylum *Proteobacteria*, including genera *Pseudomonas* and *Methylobacterium*, was the primary bacterial populations in contaminated soil from a chemical factory, and that the phylum *Proteobacteria* could produce carbon from hexachlorobenzene via relevant biodegradation/biotransformation pathways (Uhlik et al., 2014). Moreover, the genera affiliated to *Pseudomonadaceae* and *Comamonadaceae* families, belonging to the phylum *Proteobacteria,* predominated in the influent of coking wastewater, and were discovered to be with high versatility as aromatic degraders for coking wastewater contaminants (Hassan et al., 2019). Therefore, the observed environmental stress responses might be due to changes in relative abundances of phyla *Proteobacteria* in PS-exposed seawater microbiota in this study.

Bacteroidetes encode an average of 137.1 polysaccharide lyase and glycoside hydrolases per genome compared to 22.6 per genome in *Proteobacteria*, and approximately 80% of enzymes produced by *Bacteroidetes* are extracellularly located and available for polysaccharide degradation into organic acids, such as propionate and lactate (El Kaoutari et al., 2013; Louis and Flint 2017; Requena et al., 2018). *Bacteroidetes* also exert vital roles in proteolysis and organic acid formation from the degradation of peptides and amino acids (Louis and Flint 2017). On the other hand, *Proteobacteria*, as front-line responders, are sensitive to environmental factors, and the outgrowth of *Proteobacteria* is recommended as a potential marker for bacterial dysbiosis and diseases (Shin et al., 2015). It is reported that *Proteobacteria* enrichment is often a signature of bacterial dysbiosis during metabolic disorders, and that α-and γ-*Proteobacteria* correlates with severe hepatic histological features, such as ballooning degeneration, lobular and portal

inflammation, non-alcoholic steatohepatitis and liver fibrosis (Shin et al., 2015; Sookoian et al., 2020). As expected in this study, we discovered that PS-induced changes in relative abundances of bacterial taxa belonging to phyla *Proteobacteria* and *Bacteroidetes* were highly correlated with those in marine medaka liver.

We further discovered that 8 bacterial taxa belonging to phyla *Proteobacteria* and *Bacteroidetes* could be used as the separate potential biomarker for assessing PS-induced seawater microbial dysbiosis and metabolic responses of marine medaka liver. This study shows that 16S rRNA gene sequencing is an applicable and feasible tool for evaluating microbial dysbiosis in water bodies induced by microplastics. In addition, this study provides new ideas and tools for assessing the health status of organisms at higher trophic levels through the water microbial dysbiosis, which is especially important for evaluating the health status of rare aquatic organisms and those that cannot be easily found. Because it is usually infeasible to directly obtain tissue and/or body fluid samples from rare aquatic organisms and those hard to be found, at this time, the health status of target organisms can be evaluated and/or monitored through the detection of changes in microbial balance in their excreta and/or relevant environmental samples from the habitat, such as water, soil, and plants.

Due to its cost-effectiveness, acceptable resolution, and relatively low complexity of sequencing data, 16S rRNA gene sequencing has become one of the most commonly used techniques for investigations on microbial composition and function (Bokulich et al., 2020). In this study, we found that PS exposure induced metabolic dysfunction of microbes in surrounding seawater according to the functional analysis based on 16S rRNA data. However, the limitations of 16S rRNA gene sequencing remain noteworthy, and related deficiencies may lead to bias in the interpretation of microbial composition and function. Since 16S rRNA gene sequencing focuses on a limited number of universal genes, rather than on obtaining all the genes of the microbial population under study, as metagenomics does, it is unable to discover novel taxa, distinguish between microbial strains, and directly identify metabolic and function profiles of microbes (Bokulich et al., 2020; Langille et al., 2013). In addition, various potential errors involving in all steps of 16S rRNA gene sequencing from sample collection to bioinformatic analysis may produce false-positive and/or false negative screening results (Galan et al., 2016).

Given that 16S rRNA data cannot directly reflect the metabolic function of microbes, we investigated PS-induced metabolic dysfunction of surrounding seawater microbiota by employing the PICRUSt method, which was demonstrated to be able to accurately and efficiently capture functional profiles of microbial communities based on marker gene sequences of metagenomics and 16S rRNA data (Langille et al., 2013). Additionally, considering that each method has its own advantages and disadvantages, the application of multi-omics techniques, such as integrating 16S rRNA gene sequencing with transcriptomics, proteomics and/or metabolomics will enable us to observe changes in microbial function under various environmental conditions more completely (additionally from the perspective of initiation, process and/or outcome of relevant molecular events) and objectively. In this study, we integrated 16S rRNA data with the metabolomic data from marine medaka liver, and found that PS had great effects on the metabolic balance of the microecology around PS (Ye et al., 2021). Moreover, regulation of microbial function, such as overexpression and/or knockout of target gene/protein expression, and microbial community transplantation, will enable us to further identify and exploit microbial function to reduce or even eliminate the health hazards caused by environmental pollutants.

5. Conclusions

We comprehensively investigated PS-induced changes in bacterial structure and functions in the surrounding seawater cultivating marine medaka in this study. We discovered that 26 bacterial taxa belonging to phyla *Proteobacteria* and *Bacteroidetes* were significantly altered in surrounding seawater exposed to PS, of which 8 bacterial taxa could be used as potential biomarkers for assessing the microbial dysbiosis and metabolic responses of marine medaka to PS exposure. Functional analysis indicated that photosynthesis, carbon metabolism, lipid synthesis, and nucleotide metabolism were inhibited, while environmental stress responses were activated in surrounding seawater microbiota in response to PS exposure. This study provides novel insights into the effects of microplastics on microbial dysbiosis in surrounding seawater, and suggests potential roles of biomarkers related to microbial dysbiosis in the assessment of microplastic ecotoxicology, microbial dysbiosis, and the health status of organisms at higher trophic levels.

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. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2021.106724.

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