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Research Article

Effects of *Chlorella pyrenoidosa* Supplementation on Water Quality, Shrimp Growth Performance, and Biofilm Bacterial Community Structure in *Litopenaeus vannamei* Aquaculture Systems

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Chlorella pyrenoidosa is a widely used microalgae in the aquaculture process of *Litopenaeus vannamei*. In order to evaluate the effects of supplementing *C. pyrenoidosa* on water quality, shrimp growth performance, and biofilm bacterial community structure in *L. vannamei* aquaculture systems, *C. pyrenoidosa* suspension was supplemented in the precultured biofilm (PB) and water exchange (WE) culture systems. The results showed that supplementation with *C. pyrenoidosa* significantly increased the turbidity and the concentrations of total ammonia nitrogen (TAN) and nitrite nitrogen (NO_2^-—N) in the PBC system (the PB system supplemented with *C. pyrenoidosa*), but had no significant effect on those in the WEC system (the WE system supplemented with *C. pyrenoidosa*). However, the TAN concentration of all systems remained at a low level (<0.1 mg/L) throughout the experiment. Additionally, supplementation with *C. pyrenoidosa* decreased the final concentrations of total phosphorus in the PBC and WEC systems by 15.74% and 23.33%, respectively. With the supplementation of *C. pyrenoidosa*, the final weight, final biomass, and average daily growth rate of *L. vannamei* increased, while the feed conversion ratio decreased. 16srRNA high-throughput sequencing results showed that the supplementation of *C. pyrenoidosa* to the PBC system resulted in a decrease in the relative abundances of Proteobacteria and Bacteroidetes and an increase in the relative abundance of Acidobacteria. Compared to those in the initial biofilm sample, the relative abundances of nitrifying bacteria, *Nitrosococcus*, *Nitrosomonas*, *Nitrococcus*, and *Nitrospira*, in PBCF (biofilm in the PB systems with *C. pyrenoidosa*) and PBOF (biofilm in the PB systems without *C. pyrenoidosa*) decreased from 10.18% to 3.05% and 0.75%, respectively. In conclusion, *C. pyrenoidosa*, as a supplement to the aquaculture systems, has practical application prospects.

1. Introduction

Pacific white shrimp (*Litopenaeus vannamei*) is one of the most important aquaculture species in the world. The aquaculture production of *L. vannamei* in 2020 reached 5.81 million tons and increased by 52.8% compared to the production in 2015 [1]. However, the development of water exchange (WE) aquaculture also faces environmental pollution problems resulting from the discharge of a large amount of nitrogen and phosphorus nutrients, so zero WE aquaculture modes, such as recirculating aquaculture systems (RAS), have gradually been developed.

The main harmful substances in intensive aquaculture are ammonia and nitrite. The common way to remove ammonia and nitrite in RAS is through a nitrification process carried out by nitrifying microorganisms that can rely on self-produced extracellular polymeric substances to form a protective layer over bacterial colonization on the surface of substrates [2, 3]. As the specific growth rate of nitrifying microorganisms is much

slower than that of heterotrophic bacteria, the precultivation of nitrifying biofilm is an effective means for increasing the relative abundance of nitrifying bacteria and reducing the start-up period in aquaculture systems [4, 5]. Substrates are needed for the formation of nitrifying biofilms, and polymer organic synthesis substrates have been widely used because they can be customized their microshapes, individual size, and other characteristics according to the needs of practical application. Polyurethane sponges have been used as biofilm substrates in aquaculture and wastewater treatment due to their large specific surface area to provide growth space for autotrophic and heterotrophic bacteria and stable chemical characteristics to avoid the precipitation of toxic substances on microorganisms and aquatic products [4, 6–8]. Song et al. [9] reported that polyurethane sponges with biofilms controlled the concentrations of ammonia, nitrite, and suspended particulate at safe levels in zero WE systems for *L. vannamei* because of continuous nitrification activity and adsorption–desorption capacity.

The problem of constant nitrate accumulation in RAS is caused by nitrification processes. However, supplementation of different species of microalgae in feed can not only assimilate inorganic nitrogen to form organic matter through photosynthesis but also enhance immune function, resistance to infectious diseases, and tolerance to environmental stress [10]. When microalgae are cocultured with shrimp, the availability of nitrogen and phosphorus and the growth performance of shrimp are improved because microalgae become an additional food source for shrimp [11]. When live and dietary *Haematococcus pluvialis* are directly supplemented in the WE system, the growth performance of shrimp is also improved, and the relative abundance of Proteobacteria in the system is also increased [8, 12]. The extra energy input from *Platymonas* sp. in the biofloc technology system (BFT) significantly reduces the addition of carbon sources and increases the relative abundance of Proteobacteria at the phylum level and *Alteromonas* at the genus level [13]. However, due to the competition for ammonia nitrogen between nitrifying bacteria and microalgae, the nitrification process is inhibited under the condition of coculture with low ammonia nitrogen concentration [14, 15]. Therefore, the effects of microalgae on water quality and bacterial community structure need to be further studied in practical aquaculture systems.

Chlorella pyrenoidosa, a typical representative of green microalgae, has been employed to treat different types of wastewater due to its great cell reproductivity and adaptability, and this species is regarded as the most promising species for this purpose [16–18]. The high concentrations of ammonia or nitrate in wastewater are effectively converted into organic biomass through the mixotrophic photofermentation of *C. pyrenoidosa* [6, 17, 18]. *C. pyrenoidosa* has the potential to be used in aquaculture to supplement protein sources, as its protein content can reach 52.4% [19]. What is more, the growth of *C. pyrenoidosa* is promoted due to mixed nitrogen sources containing ammonia and nitrate in the long term or the increase in substrate surface area [20, 21]. *C. pyrenoidosa* also impacts bacterial community structure, as Bhatt et al. [16] reported that 96% of *Enterobacteriaceae* and 98% of *Salmonella* sp. in real sewage were removed because they were attached or entrapped within the *C. pyrenoidosa* exopolysaccharides. Feeding *C. pyrenoidosa* can significantly affect the diversity and abundance of bacteria in the surrounding water and gut of *Hyriopsis cumingii*, including a significant increase in the relative abundance of *Cetobacterium* [22].

In this study, we investigated the changes in water quality, shrimp growth performance, and bacterial community of biofilm by supplementing *C. pyrenoidosa* to precultured biofilm (PB) and WE aquaculture systems of *L. vannamei*. The study provides a theoretical basis reference for the practical application of *C. pyrenoidosa* in the aquaculture systems of *L. vannamei*.

2. Materials and Methods

2.1. Cultivation Method of C. pyrenoidosa. *C. pyrenoidosa* (FACHB-9) was purchased from the Freshwater Algae Culture Collection of the Institute of Hydrobiology (Wuhan, China) and was cultured in a shaking incubator (MQD-B2R, Shanghai Minquan Instrument Co., Ltd., China) using BG-11. The BG-11 medium consisted of glucose (35 g/L), citric acid (0.006 g/L), ferric ammonium citrate (0.006 g/L), ethylenediamine tetraacetic acid (EDTA) (0.001 g/L), K_2HPO_4 (0.15 g/L), $MgSO_4 \cdot 7H_2O$ (0.0756 g/L), $CaCl_2 \cdot 2H_2O$ (0.036 g/L), Na_2CO_3 (0.02 g/L), $NaNO_3$ (1.45 g/L), and trace element solution (1 mL/L) in deionized water. The trace element solution consisted of H_3BO_3 (2.86 g/L), $MnCl_2{\cdot}4H_2O$ (1.81 g/L), $ZnSO_4{\cdot}7H_2O$ (0.222 g/L), $Na_2MoO_4{\cdot}2H_2O$ (0.39 g/L), $CuSO_4{\cdot}5H_2O$ (0.079 g/L), and $Co(NO_3)_2{\cdot}6H_2O$ (0.049 g/L) in deionized water. The culture conditions of the first stage for the two stages were set as follows: inoculum of 10%, temperature of 30°C, shaker speed of 150 r/min, 12/12 hr of light/dark, light intensity of 4,000 lx, and culture time of 72 hr. The second stage conditions involved autotrophic culture coupled without glucose, and the light did not exceed 20,000 lx. The cell concentration of *C. pyrenoidosa* was not less than 1% after inoculation and was counted by the erythrocytometry and hot ethanol methods in the early and later stages, respectively. The chlorophyll-*a* concentration of *C. pyrenoidosa* in this study was 8.765 mg/L at the end of the culture.

2.2. Cultivation Method of Polyurethane Sponge Biofilm. First, 0.024 m^3 of polyurethane sponge (specification: $2 \times 2 \times 2$ cm^3, porosity: 98%) was placed in a round bucket containing 0.080 m^3 of artificial seawater at 18 practical salinity units (psu), which contained 1% (V/V) nitrifying bacteria preparation (Qingdao Seadoctor Co., Ltd., Qingdao, China), 0.1% (V/V) trace element solution and 0.01% (W/V) yeast extract. The polyurethane sponge floated freely in seawater. The trace element solution consisted of $ZnSO_4{\cdot}7H_2O$ (0.287 mg/L), $CuSO_4{\cdot}5H_2O$ (7.5 mg/L), $MnCl_2{\cdot}4H_2O$ (14.85 mg/L), $FeCl_3$ (5380 mg/L), $Na_2MoO_4{\cdot}2H_2O$ (6.8 mg/L), EDTA (2.4 mg/L), $CoCl_2 \cdot 6H_2O$ (12 mg/L), and $NiSO_4$ (2.4 mg/L). Second, 4 g of $NaNO_2$ was added to make the NO_2^-—N concentration reach 10 mg/L. When the NO_2^-—N concentration was lower than 0.05 mg/L, 4 g of $NaNO_2$ was added, and the process was repeated five times. Finally, 0.31 g of NH_4Cl was added to make the TAN concentration reach 10 mg/L, and the cultivation of the biofilm was completed when the TAN concentration was lower than 0.05 mg/L. The temperature and DO were

controlled at 27–29°C and 4.5–6.0 mg/L in the process of biofilm cultivation, respectively.

2.3. Experimental Shrimp Stocking and Management. Twelve rectangular plastic tanks ($0.75 \times 0.4 \times 0.4 = 0.12\ m^3$) were selected as culture tanks and cleaned with sterilized water after being disinfected with 5% hydrochloric acid solution. Then, they were filled with 80 L of artificial seawater at 15 psu. Aeration stones and heating rods were placed in the tanks to control the temperature at approximately 27.5°C and the dissolved oxygen (DO) at 7.5 mg/L before the experiment.

Two aquaculture systems were constructed, namely, the PB systems and WE systems; the systems in which 10 mL of *C. pyrenoidosa* was supplemented every 72 hr were denoted as PBC and WEC, respectively, and the remaining systems were denoted as PBO and WEO. The PB systems were individually filled with $0.004\ m^3$ of sponge biocarriers, which accounted for 5% of the culture water volume and were placed in a 2 mm mesh-diameter Terylene bag. The bag was suspended directly above the culture tank and completely submerged throughout the culture process. Aeration stones were placed inside the bags to prevent internal oxygen deficiency, and sponges were cleaned once a week to restore the physical adsorption capacity of suspended solids.

L. vannamei was purchased from Guangdong Haida Group Co., Ltd. (Guangzhou, China). In the laboratory, they were acclimatized in two 80 L tanks with artificial seawater (salinity at 15 psu, temperature at 27.5°C and pH at 8.15). After 20 days of temporary culture, shrimp larvae with an average body weight of 0.1 ± 0.001 g and an average body length of 1.5 ± 0.1 cm were selected for the experiment. Sixty-four shrimp larvae were cultured in each tank (800 shrimp/m^3). Shrimp were fed four times a day (7.00 a.m., 12 a.m., 4.00 p.m., and 9.00 p.m.) with commercial feed (crude protein ≥ 50%, Shenzhen Aohua Group Co., Ltd.). The feeding amount was adjusted according to the biomass in the systems. During the experiment, the PBC and WEC systems had 10 mL of *C. pyrenoidosa* added every 3 days. On the second day of the experiment, a nitrifying bacteria preparation containing ammonia-oxidizing microorganisms and nitrite-oxidizing bacteria (NOB) was added to every tank. Volatilized water was added to the PB systems every day. The WE systems were exchanged with 16 L (20%) of fresh seawater every 3 days.

2.4. Determination of Physicochemical Parameters of Water. The parameters of all aquaculture systems were measured daily during the morning between 8:00 and 9:00 for 79 days. The water temperature, pH, and salinity were measured directly using a mercury thermometer, a digital pH meter (pH 610, Wiggens Company, Germany), and a mercury pycnometer, respectively. DO and turbidity were measured by a portable hand-held DO meter (HQ30D, Hach Company, USA) and portable turbidimeter (2100P, Hach Company, USA), respectively.

The concentrations of total ammonia nitrogen (TAN) and nitrite nitrogen (NO_2^-—N) were determined by Nessler reagent colorimetry and N—(1-naphthyl)-ethylenediamine spectrophotometry every 12 hr, respectively. The concentration of nitrate nitrogen (NO_3^-—N) was determined by naphthylethylenediamine hydrochloride spectrophotometry, and total phosphorus (TP) was determined by Mo-Sb antispectrophotometry every 72 hr.

2.5. Growth Performance. L. vannamei was harvested after draining the tanks. The mean final weight, survival, final biomass, feed conversion ratio (FCR), and average daily growth rate (ADG) of the shrimp were determined according to Hoang et al. [23].

2.6. Scanning Electron Microscope (SEM) Method. On the 79th day of the experiment, two equal portions of polyurethane sponges were taken from the systems of PBC and PBO. The samples without any treatment were denoted as PBCA and PBOA, and the samples cleaned several times with sterilized water were denoted as PBCF and PBOF. Four groups of polyurethane sponges were also analyzed by scanning electron microscopy (VEGA3, Tescan, Czechia).

2.7. 16srRNA Sequencing Method. On Day 79 of the experiment, polyurethane sponges were randomly selected from the systems of PBC and PBO and washed several times with sterilized water to obtain the suspension of adsorbate. The suspension was centrifuged, and the supernatant was removed to obtain the adsorbate samples, which were successively denoted by PBCA and PBOA. The cleaned polyurethane sponges were cut and immersed in sterilized water again. Then, they were placed in ultrasonic cleaners (QTSXR20500, Tianjin Ruipu Electronic Instrument Company, China) for 15 min to obtain a biofilm suspension. The suspension was centrifuged, and the supernatant was removed to obtain the biofilm samples, which were successively denoted by PBCF and PBOF. Additionally, the initial biofilm sample of precultured polyurethane sponges was denoted by initial biofilms in sponges (IBF).

Total DNA was extracted from the above samples using an E.Z.N.A. Soil DNA Kit (OMEGA Bio-tek, Norcross, USA) according to the kit instructions. 16srRNA sequencing was performed using the MiSeq sequencing system of the Illumina platform (Shanghai Meiji Biotechnology Co., Ltd., Shanghai, China). Qiime software was used to eliminate sequences with a length of less than 150 bp and chimeras in the original sequence, and then high-quality sample sequences were obtained. The Uparse platform was used to cluster the high-quality sequences at the 97% similarity level to obtain the operational taxonomic units(OTUs) table. The sequence with the highest richness was selected as the representative sequence of the OTUs, and the ribosomal database project classifier Bayesian algorithm was used to count the community species composition in the OTUs representative sequence. The comparison library was Silva138/16s_bacteria. The alpha and beta diversity analyses were carried out by the Shengxinyun platform (https://www.majorbio.com).

2.8. Data Analysis. A series of one-way analysis of variances (ANOVAs) were performed, followed by Fisher's least significant difference test to determine the differences among the systems for water quality parameters and the growth parameters of shrimp ($p < 0.05$). Data are expressed as the mean ± standard deviation. All statistical analyses were conducted using SPSS, version 26.0.

Table 1: Physicochemical parameters of the water in the different *L. vannamei* aquaculture systems.

Parameters	PBC	PBO	WEC	WEO
Temperature (°C)	27.5 ± 0.1^{a} (26.3–28.1)	27.4 ± 0.1^{a} (26.4–28.0)	27.7 ± 0.1^{a} (26.5–28.4)	27.8 ± 0.1^{a} (26.7–28.5)
Salinity (%)	14.94 ± 0.14^{a} (14.75–15.31)	15.02 ± 0.16^{a} (14.67–15.45)	14.98 ± 0.12^{a} (14.45–15.61)	14.99 ± 0.15^{a} (14.53–15.64)
DO (mg/L)	7.56 ± 0.02^{a} (7.10–7.93)	7.50 ± 0.02^{a} (7.19–7.70)	7.52 ± 0.01^{a} (7.34–7.70)	7.48 ± 0.01^{a} (7.21–7.68)
pH	7.87 ± 0.04^{b} (7.45–8.32)	7.80 ± 0.03^{b} (7.36–8.34)	8.09 ± 0.03^{a} (7.88–8.35)	8.09 ± 0.03^{a} (7.83–8.35)^{a}
Turbidity (NTU)	5.96 ± 0.20^{b} (1.25–13.45)	1.15 ± 0.02^{c} (0.93–1.63)	13.49 ± 0.23^{a} (1.25–26.85)	11.27 ± 0.36^{a} (1.19–22.25)
TAN (mg/L)	0.055 ± 0.003^{a} (0.007–0.078)	0.015 ± 0.001^{b} (0.004–0.022)	0.065 ± 0.001^{a} (0.009–0.095)	0.067 ± 0.001^{a} (0.008–0.096)
NO_2^-—N (mg/L)	2.19 ± 0.07^{b} (0.01–5.02)	0.34 ± 0.01^{c} (0.01–0.69)	4.47 ± 0.02^{a} (0.01–8.09)	4.54 ± 0.13^{a} (0.01–7.79)
NO_3^-—N (mg/L)	46.60 ± 2.02^{a} (2.24–84.98)	52.85 ± 2.08^{a} (2.25–95.04)	13.83 ± 1.06^{b} (2.21–32.85)	10.23 ± 0.70^{b} (2.20–22.30)
TP (mg/L)	0.23 ± 0.02^{ab} (0.01–0.57)	0.27 ± 0.02^{a} (0.01–0.66)	0.12 ± 0.01^{b} (0.01–0.322)	0.15 ± 0.01^{ab} (0.01–0.42)

Note: Mean values in the same row with different superscripts differ significantly ($p<0.05$). Abbreviations: PBC, system with precultured biofilm and *C. pyrenoidosa*; PBO, system only with precultured biofilm; WEC, system with water exchange and *C. pyrenoidosa*; WEO, system only with water exchange. Values are the means of three replicates ± standard deviations.

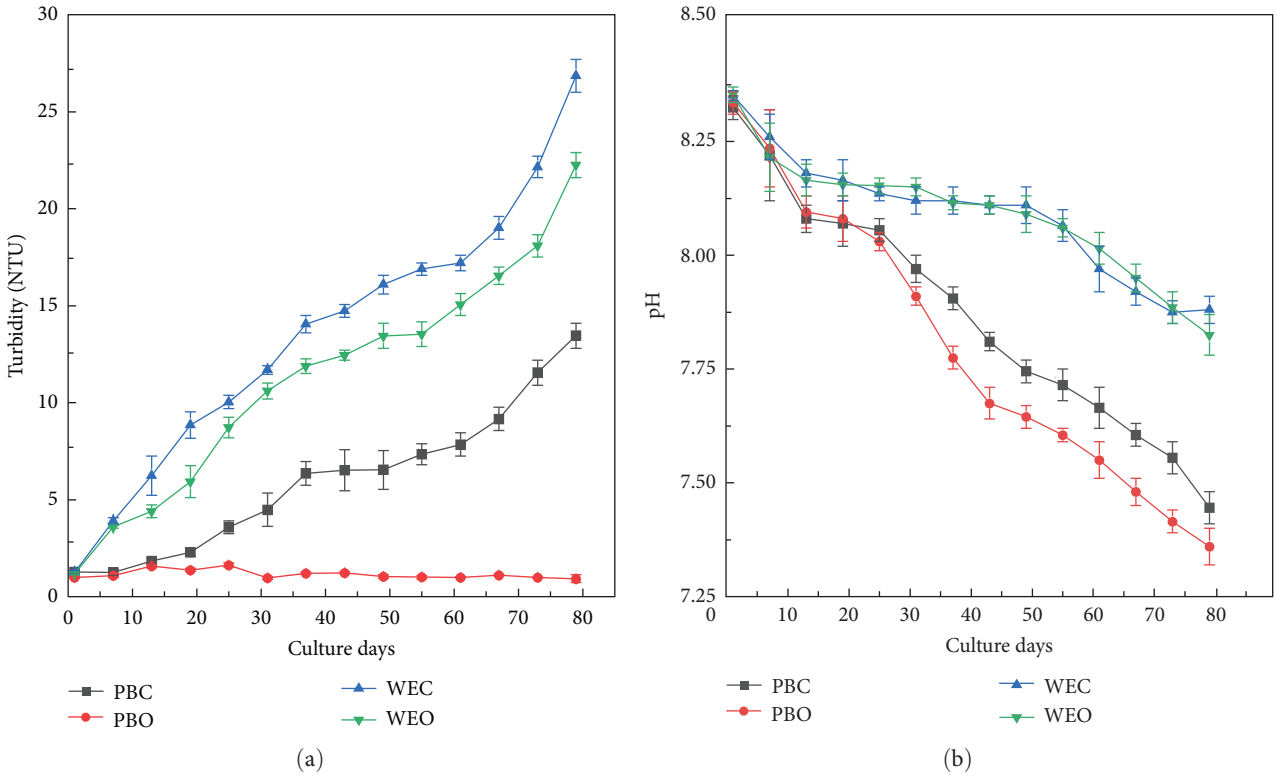


Figure 1: Variation in the turbidity (a) and pH (b) in the *L. vannamei* aquaculture systems (PBC, system with precultured biofilm and *C. pyrenoidosa*; PBO, system only with precultured biofilm; WEC, system with water exchange and *C. pyrenoidosa*; WEO, system only with water exchange). Data are presented as the mean ($n=3$). Error bars indicate standard error.

3. Results

3.1. Water Quality Parameters. The water quality parameters of the experimental systems are presented in Table 1. The one-way ANOVA test did not show significant differences in temperature, salinity, or DO ($p>0.05$). As shown in Figure 1 (a), the turbidity in the WE systems was significantly higher than that in the PB systems ($p=0.007$). With the supplementation of *C. pyrenoidosa* to the PBC system, the turbidity significantly increased ($p=0.014$). *C. pyrenoidosa*, a freshwater alga, cannot accumulate in large quantities with seawater. In this study, the gradual increase in turbidity of the PBC system was caused by continuous supplementation with *C. pyrenoidosa*. The pH values of all systems decreased continuously, as shown in Figure 1(b), and reached a minimum at the end of the experiment. The pH values were significantly different between the PB and WE systems ($p=0.020$). Supplementation with *C. pyrenoidosa* had no effect on pH change ($p=0.444$, $p=0.949$).

The concentration of TAN continued to rise during the initial culture phase, and all systems except the PBO system

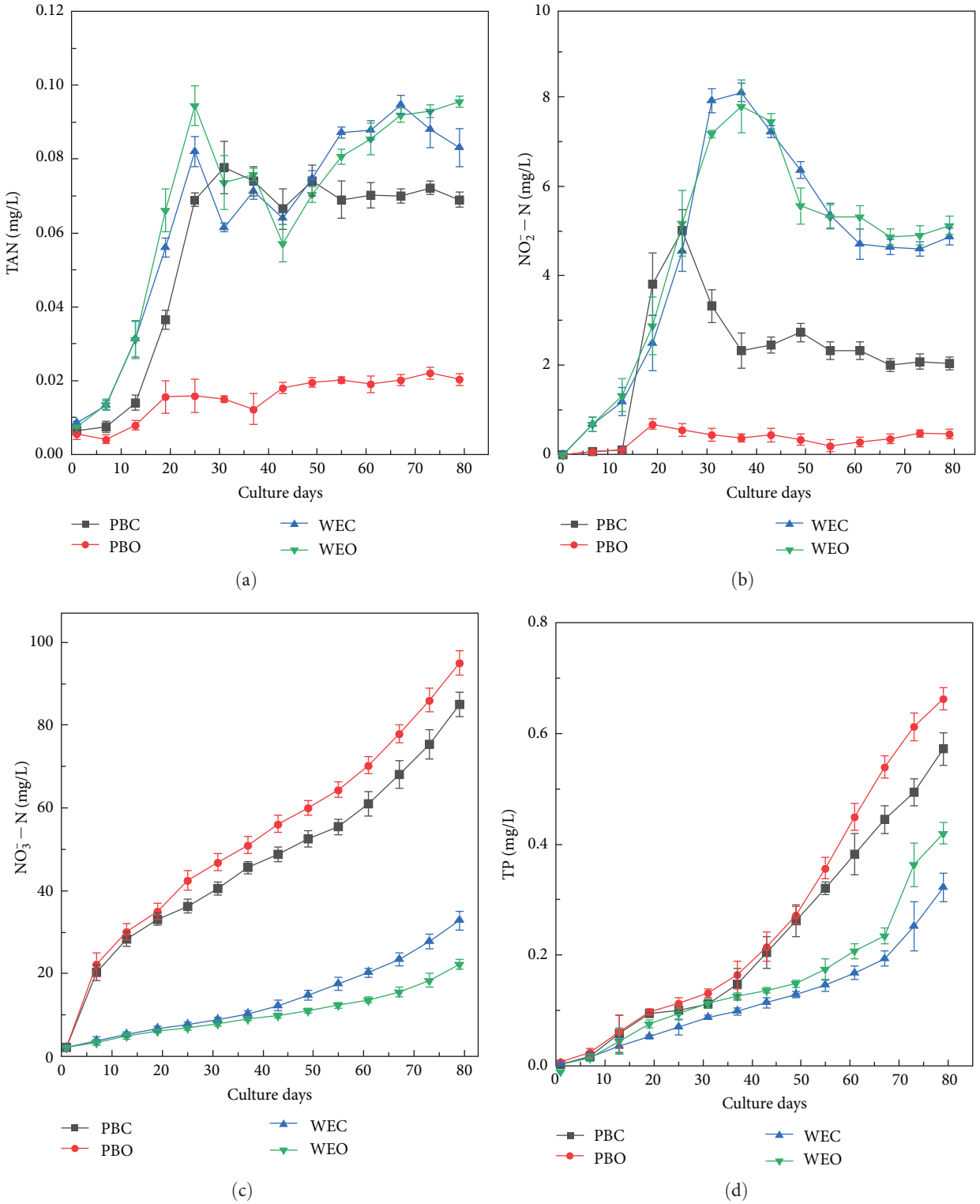


FIGURE 2: Concentration variation in the TAN (a), NO_2^-—N (b), NO_3^-—N (c), and TP (d) in the *L. vannamei* aquaculture systems (PBC, system with precultured biofilm and *C. pyrenoidosa*; PBO, system only with precultured biofilm; WEC, system with water exchange and *C. pyrenoidosa*; WEO, system only with water exchange). Data are presented as the mean ($n = 3$). Error bars indicate standard error.

reached peak TAN concentrations on days 19–25 (Figure 2(a)); however, the TAN concentration of all systems remained at a low level (<0.1 mg/L) throughout the experiment. The concentration of TAN in the WEO system was significantly higher than that in the PBO system ($p < 0.001$). With the supplementation of *C. pyrenoidosa*, the concentration of TAN in the PBC system significantly increased ($p < 0.001$), while the concentration of TAN in the WEC system was not significantly different from that in the WEO system ($p = 0.851$). The concentration of NO_2^-—N continued to rise during the initial culture phase and

Table 2: Growth parameters of *L. vannamei* in the different aquaculture systems.

Parameters	PBC	PBO	WEC	WEO
Mean final weight (g)	14.93 ± 0.12^{a}	12.53 ± 0.05^{b}	14.03 ± 0.05^{a}	12.10 ± 0.08^{b}
Survival (%)	80.21 ± 1.95^{a}	83.33 ± 0.74^{a}	68.75 ± 1.28^{b}	58.85 ± 0.74^{c}
Final biomass (kg/m^3)	9.58 ± 0.16^{a}	8.36 ± 0.11^{b}	7.72 ± 0.15^{c}	5.70 ± 0.04^{d}
FCR	1.29 ± 0.02^{d}	1.48 ± 0.02^{c}	1.60 ± 0.03^{b}	2.16 ± 0.02^{a}
ADG (g/day)	0.189 ± 0.002^{a}	0.159 ± 0.001^{b}	0.178 ± 0.001^{ab}	0.153 ± 0.001^{d}

Note: Mean values in the same row with different superscripts differ significantly ($p<0.05$). Abbreviations: PBC, system with precultured biofilm and *C. pyrenoidosa*; PBO, system only with precultured biofilm; WEC, system with water exchange and *C. pyrenoidosa*; WEO, system only with water exchange. Values are the means of three replicates ± standard deviations.

peaked on days 25–37 in all systems (Figure 2(b)). Although the WE systems exchanged water several times, the concentrations of NO_2^-—N were still significantly higher than those in the PB systems ($p = 0.003$). With the supplementation of *C. pyrenoidosa*, the concentration of NO_2^-—N in the WEC system did not change significantly ($p = 0.929$), but that in the PBC system increased significantly ($p = 0.014$).

The concentrations of NO_3^-—N (Figure 2(c)) and TP (Figure 2(d)) continued to increase in all systems. Due to WE in the WE systems, the concentrations of NO_3^-—N in the WE systems were significantly lower than those in the PB systems ($p<0.001$). With the supplementation of *C. pyrenoidosa* to the PBC and WEC systems, the final concentrations of TP decreased by 15.74% and 23.33%, respectively ($p = 0.013$, $p = 0.003$). In contrast, the final concentration of NO_3^-—N in the PBC system decreased by 10.58%, while that in the WEC system increased by 47.36% ($p = 0.044$, $p = 0.012$).

3.2. Growth Performance of L. vannamei. The growth performance of *L. vannamei* in different systems is shown in Table 2. The survival and final biomass in the PB systems were significantly higher than those in the WE systems ($p<0.001$, $p<0.001$), and the final biomass in the PBC system was highest. With the supplementation of *C. pyrenoidosa*, the mean final weight ($p<0.001$, $p<0.001$) and ADG ($p = 0.003$, $p = 0.023$) increased significantly, and the FCR decreased significantly ($p<0.001$, $p<0.001$).

3.3. Analysis of Microbial Community Structure

3.3.1. Morphology of Adsorbates and Biofilms of Polyurethane Sponges. The SEM images of the polyurethane sponges in the PB systems with different treatments are shown in Figure 3. The polyurethane sponges had porous network structures and were permeated with pores of similar diameter, which improved the specific surface area of the sponges and increased the contact area between the water and sponges. These characteristics were conducive to the attachment growth and reproduction of microorganisms. The surfaces of the polyurethane sponges were covered by a large number of microorganisms, as can be observed in all images. These microorganisms grew on the surfaces of the sponges in the form of bacterial micelles. By comparing the SEM images before and after cleaning, it was found that a large amount of solids were adsorbed on the surface of polyurethane sponges and could be removed by squeezing polyurethane sponges to reduce turbidity. Additionally, abundant residues of *C. pyrenoidosa* were observed on the surfaces of the polyurethane sponges in the PBC system.

3.3.2. Analysis of the Bacterial Community Structure of the Adsorbates and Biofilms. A total of 950 OTUs were generated from 237,795 filtered sequence reads. The alpha diversity indices of the bacteria and OTU numbers in the biofilms and adsorbates are shown in Table 3. The coverages of all the samples were above 99%, indicating that all the sequences in the samples had been detected. The Chao1 index, Shannon index, and ACE index in PBOF and PBCF were all higher than those in IBF ($p<0.001$). The Chao1 index, Shannon index, and ACE index of the adsorbates were all lower than those of the biofilms, while the Simpson index of the adsorbates was higher than that of the biofilms ($p<0.001$). These results indicated that the diversity and richness of the bacterial community in the adsorbates were lower than those in the biofilms.

The phyla with a relative abundance higher than 1% are shown in Figure 4. Twenty-nine phyla were detected in the five samples. The phyla included Proteobacteria, Bacteroidetes, Acidobacteria, Chloroflexi, Planctomycetota, Desulfobacterota, Dadabacteria, etc. Compared with those in the initial biofilm sample, the relative abundances of Proteobacteria and Dadabacteria in PBOF decreased, and the relative abundances of Bacteroidota and Planctomycetota in PBOF increased ($p<0.05$). With the supplementation of *C. pyrenoidosa*, the relative abundances of Proteobacteria and Bacteroidetes in PBCA and PBCF significantly decreased, while the relative abundances of Acidobacteria and Desulfobacterota in PBCA and PBCF significantly increased ($p<0.05$). The relative abundances of Planctomycetota and Chloroflexi in the biofilms were higher than those in the adsorbates ($p<0.05$). The relative abundances of Nitrospirota in IBF, PBAO, PBOF, PBCA, and PBCF were 0.002%, 0.043%, 0.072%, 0.337%, and 2.355%, respectively. Therefore, the relative abundances of Nitrospirota in the PB systems increased compared with those in the initial biofilm sample and increased with the supplementation of *C. pyrenoidosa*.

The bacteria in the five samples were classified into 460 genera. The bacterial communities at the genus level with the top 50 relative abundances were clustered according to the taxon abundance distribution and the similarity degree between samples (Figure 5). The dominant genera included *Halomonas*, *Hyphomicrobium*, *Ruegeria*, *SM1A02*, *Acanthopleuribacter*, *Catenovulum*, *Gammaproteobacteria*, norank_f__A4b, etc. Compared with those in the initial biofilm sample, the relative abundances of *Hyphomicrobium* and *Halomonas* in PBOF decreased, and the relative abundances of *Ruegeria* and *SM1A02* increased ($p<0.05$). With the supplementation of

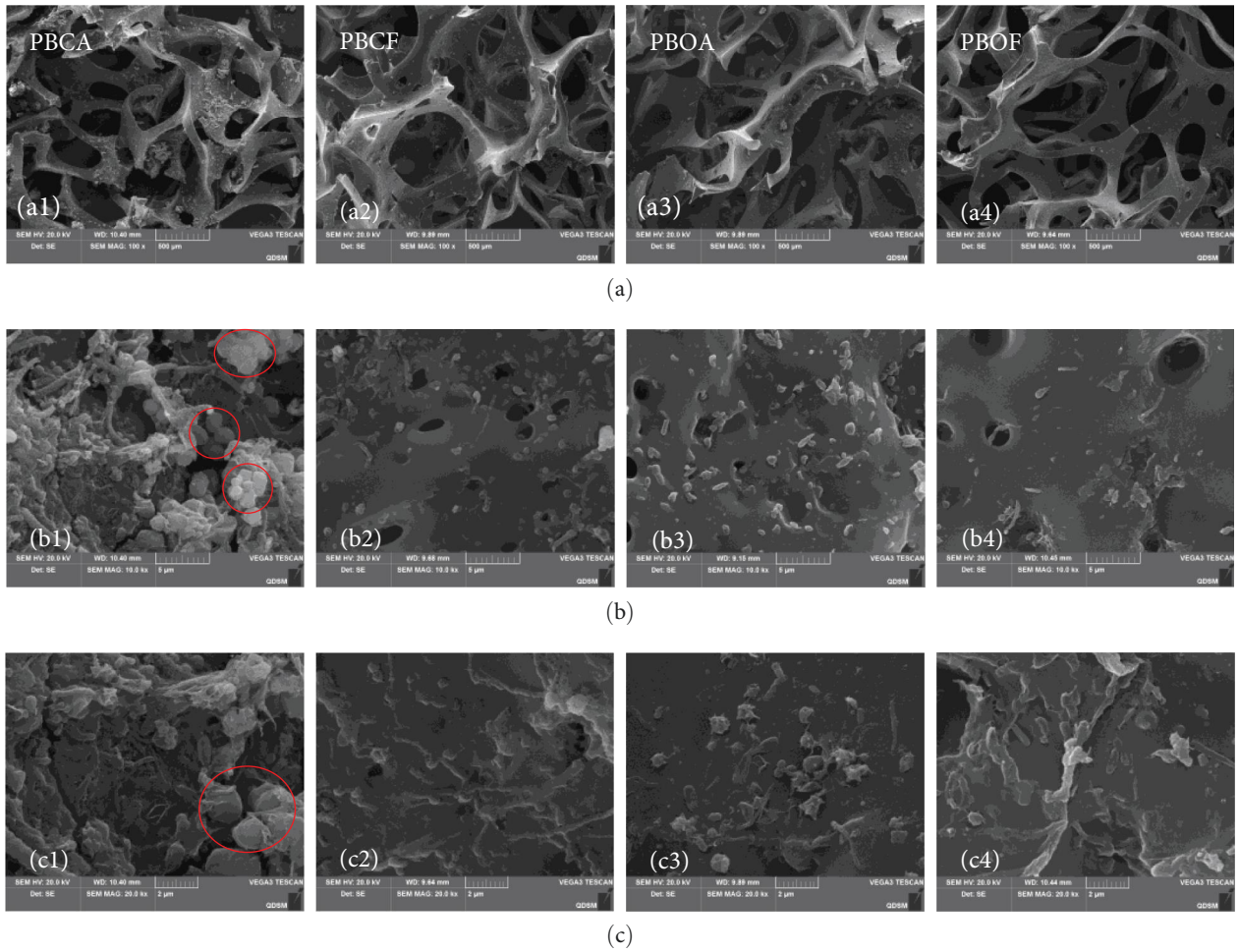


FIGURE 3: SEM images of the sponges under different treatments (PBCA, sponges in the PBC system without cleaning; PBCF, sponges in the PBC system with cleaning; PBOA, sponges in the PBO system without cleaning; PBOF, sponges in the PBO system with cleaning). Images in the same column are the same sample at different magnifications; images in the first (a), second (b), and third (c) lines are magnified 100 times, 10,000 times, and 20,000 times, respectively. *Note*. *C. pyrenoid*osa is circled in B and C in PBCA.

TABLE 3: Sequencing results and alpha diversity index of the adsorbates and biofilms in the PB systems.

Samples	OTUs	Coverage (%)	Shannon	Simpson	Ace	Chao1
IBF	366	99.91	3.80^{d}	0.0421^{c}	401.8^{c}	399.6^{c}
PBOF	586	99.75	4.78^{a}	0.0189^{e}	678.6^{a}	717.9^{a}
PBCF	603	99.80	4.24^{b}	0.0671^{b}	672.7^{a}	670.1^{a}
PBCA	417	99.75	2.24^{e}	0.3514^{a}	534.6^{b}	557.6^{b}
PBOA	512	99.78	4.11^{c}	0.0394^{d}	591.1^{b}	598.6^{b}

Note: Mean values in the same column with different superscripts differ significantly ($p<0.05$). Abbreviations: IBF, initial biofilms in sponges; PBOF, biofilms in PBO sponges; PBCF, biofilms in PBC sponges; PBCA, adsorbates in PBC sponges; PBOA, adsorbates in PBO sponges; OTUs, operational taxonomic units.

C. pyrenoidosa, the relative abundances of *Acanthopleuribacter* and *Catenovulum* in PBCA and PBCF significantly increased, while the relative abundances of *Gammaproteobacteria* and *Ruegeria* in PBCA and PBCF significantly decreased ($p<0.05$). The relative abundances of *SM1A02* and norank_f__A4b in the biofilms were higher than those in the adsorbates. In the present study, two genera of ammonia-oxidizing bacteria, *Nitrosococcus* and *Nitrosomonas*, and two genera of NOB, *Nitrococcus* and *Nitrospira*, were identified. The relative abundances of *Nitrosococcus*, *Nitrosomonas*, and *Nitrococcus* in the PBOF decreased by 4.23%, 1.16%, and 4.11%, respectively, compared with those in the IBF ($p<0.05$). Moreover, the relative abundance of *Nitrosococcus* in the IBF system was 4.23%, while that in the PB system was less than 0.01%. The relative abundance of *Nitrospira* in the PBC system was significantly higher than that in the PBO system. With the supplementation of *C. pyrenoidosa*, the relative abundance of *Nitrococcus* in PBCA and PBCF decreased, while the relative abundance of *Nitrosomonas* in PBCA and PBCF increased ($p<0.05$).

The results of the Venn diagram (Figure 6(a)) showed that the number of unique genera in IBF was the largest, and the results of the PCoA analysis (Figure 6(b)) showed that IBF was far away from other biofilm samples, indicating that there was a significant difference in the bacterial community composition on the genus level between the initial biofilm and the biofilms during the culturing period. In Figure 6(b), the obvious clusters of biofilms and adsorbates in the same system indicated that the bacterial composition of adsorbates

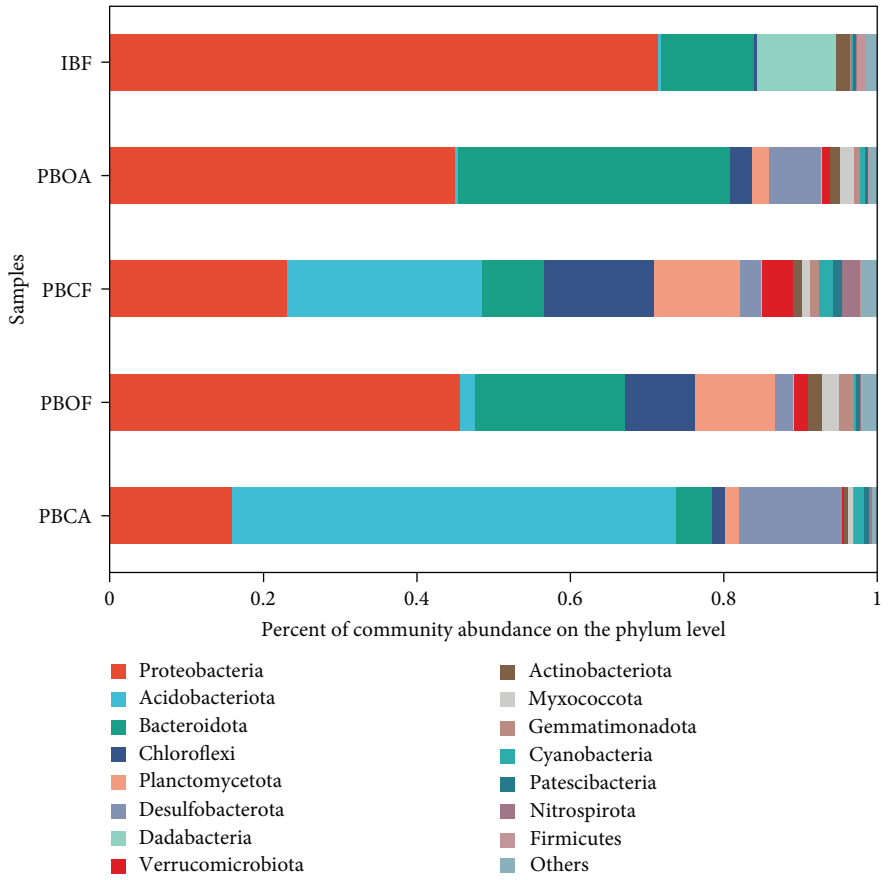


FIGURE 4: Microbial community diversity and relative abundance of the adsorbates and biofilms on the phylum level (IBF, initial biofilms in sponges; PBOF, biofilms in PBO sponges; PBCF, biofilms in PBC sponges; PBCA, adsorbates in PBC sponges; PBOA, adsorbates in PBO sponges).

and biofilms in the same system were the most similar on the genus level, and the supplementation of *C. pyrenoidosa* made them change.

4. Discussion

Ammonia and nitrite are the main targets for removal in aquaculture. The concentrations of TAN in all systems remained within the recommended range for shrimp growth, but the peak NO_2^-—N concentration values in the WE systems were higher than the recommended range [24]. The results in the PBO system showed that PB had the ability to maintain the concentrations of TAN and NO_2^-—N at safe levels due to continuous nitrification activity, which was consistent with the results of Song et al. [9]. The PB attached to the surface of polyurethane sponge pores was composed of nitrifying microorganisms, and their extracellular polymers and nitrifying microorganisms were present throughout the experiment. However, nitrification was inhibited by disadvantageous competition with algae for dissolved inorganic nitrogen or by toxic compounds released by the algae [15, 25, 26]. The increase in concentrations of TAN and NO_2^-—N in the PBC system may have been due to the cell death and rupture of *C. pyrenoidosa*, which is not adapted to the seawater environment. The simultaneous rise in turbidity and TAN and NO_2^-—N concentrations further confirm this view. Additionally, the decrease in TP was due to the assimilation of *C. pyrenoidosa*. The difference between the increase time of the TAN and NO_2^-—N concentrations and the decline time of the TP concentrations in the PB systems explained the different mechanisms by which *C. pyrenoidosa* affected them. The phosphorus in the PBC system was the main limiting factor for *C. pyrenoidosa* growth because the concentration of TP was below 0.05 mg/L, and the ratio of inorganic nitrogen to TP was above 1,000 during the first 16 days of the experiment. The increase in daily feeding weight increased the concentration of TP and decreased the ratio of inorganic nitrogen to TP. Compared with those in the PBO system, in the PBC system, the assimilation of *C. pyrenoidosa* led to a decrease in the concentrations of NO_3^-—N and TP. The concentrations of TAN and NO_2^-—N in the PBC system peaked on Day 27, and the reason for the postpeak decline was that the nitrifying microorganisms in the systems were less affected by *C. pyrenoidosa*, including the microbial community adapting to its influence or the weakening of nutrient competition [27]. In contrast to the PB systems, in the WE systems, the supplementation of *C. pyrenoidosa* had no effect on the water quality parameters. Ammonia and nitrite were removed when the water was exchanged rather than when nitrification occurred in the initial stage of the experiment.

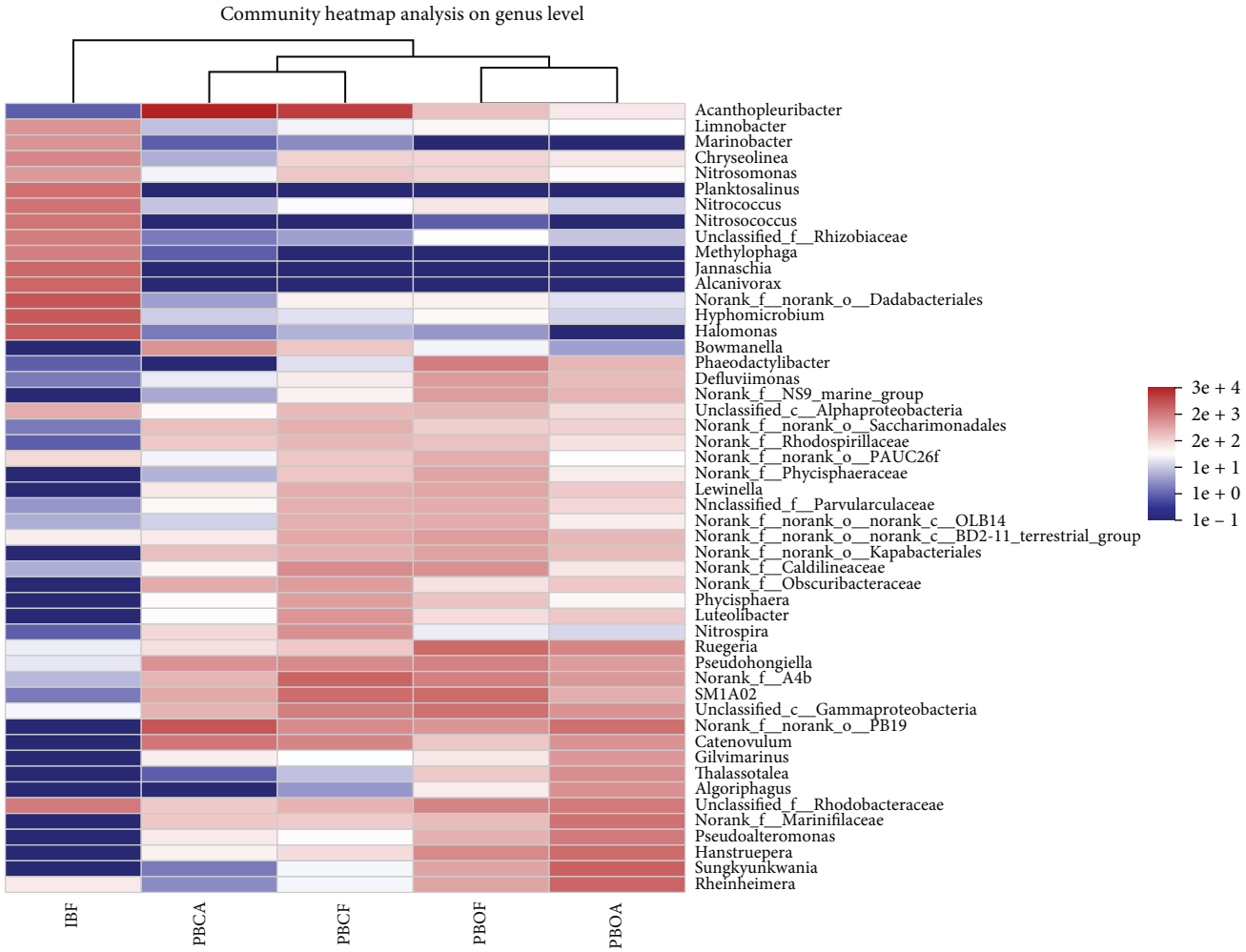
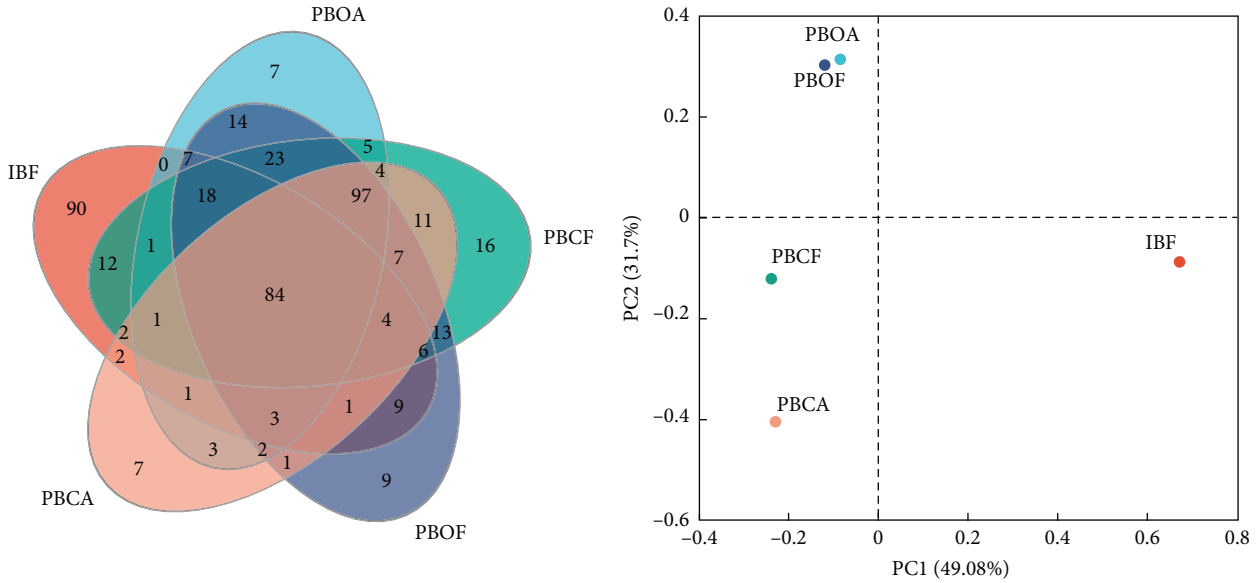


Figure 5: Community heatmap of the adsorbates and biofilms on the genus level (IBF, initial biofilms in sponges; PBOF, biofilms in PBO sponges; PBCF, biofilms in PBC sponges; PBCA, adsorbates in PBC sponges; PBOA, adsorbates in PBO sponges).

The peak of the TAN and NO_2^-—N concentrations in the WE systems may have been due to the gradual accumulation of nitrifying microorganisms. In addition, compared to the PB systems, in the WE systems, the peak value of the NO_2^-—N concentration was higher, and the peak period was delayed because the generation time of nitrifying microorganisms was much longer than those of heterotrophic bacteria [28].

Water quality parameters, including TAN, NO_2^-—N, turbidity, pH, and DO, were significantly correlated with the survival of shrimp [24, 29, 30]. The survival of shrimp in the PB systems was higher than that in the WE systems due to the optimal water quality of the PB systems. In addition, the survival in the WE systems was lower than the 69% reported by Hoang et al. [23] owing to the lower water change rate in this study. The increase in the final weight of the systems supplemented with *C. pyrenoidosa* indicated the presence of shrimp feeding on *C. pyrenoidosa*. The ADG and final biomass amount in the PBC system were the greatest due to the excellent water quality and the presence of *C. pyrenoidosa*. The results showed that FCR decreased as *C. pyrenoidosa* increased up to 50% protein and served as additional feed for shrimp [19]. FCR in the PBC system achieved the same level as the BFT reported by Flores-Valenzuela et al. [31] with supplementation of *C. pyrenoidosa* instead of a carbon source. The concentrations of NO_3^-—N and TP in the WEO system were significantly lower than those in the PBO system because of the loss of nutrients caused by WE. The results of this study showed that supplementation with *C. pyrenoidosa* decreased the nitrate concentration in the PB systems but increased the nitrate concentration in the WE systems. The reasons for the differences in the variation in nitrate concentrations between the two systems need further study because the nitrate concentration in this study was related to nitrification, denitrification, shrimp metabolism, *C. pyrenoidosa* assimilation, and biodegradation. In conclusion, supplementation with *C. pyrenoidosa* decreased the concentration of TP and improved the growth performance of *L. vannamei* in this study.

The SEM images showed that *C. pyrenoidosa* was adsorbed to the pore surface of the polyurethane sponges. Therefore, polyurethane sponges not only provided growth surfaces for bacteria but also for *C. pyrenoidosa* that tended to grow on the substrate surfaces [21]. In this study, the turbidity increase in the PBC system with *C. pyrenoidosa* and substrates was significantly higher than that in the WE system with *C. pyrenoidosa*, as the increase of substrate surface in aquaculture system would



(a) (b)

FIGURE 6: Venn diagram of the bacterial community (a) and 2D sorting diagram of the PCoA analysis (b) on the genus level (IBF, initial biofilms in sponges; PBOF, biofilms in PBO sponges; PBCF, biofilms in PBC sponges; PBCA, adsorbates in PBC sponges; PBOA, adsorbates in PBO sponges).

increase the biomass of *C. pyrenoidosa* [21]. Moreover, the residues of *C. pyrenoidosa* on the polyurethane sponge surfaces provided organic matter for heterotrophic microorganisms, which competed with nitrifying bacteria for DO as the organic matter was decomposed.

As seen from the diagrams of Venn and PCoA analysis, the intrasystem similarity was higher than the intersystem similarity, and the bacterial community structure of IBF was significantly different from that of the other samples. Microbial community structure is directly reflected by the microbial diversity index from different perspectives. The bacterial diversity of PBOF and PBCF was higher than that of IBF. Proteobacteria and Bacteroidetes were the dominant phyla in all biofilms and adsorbate samples, which are consistent with the reports of other aquaculture systems [8, 12, 13]. Proteobacteria and Bacteroidetes are the main heterotrophic bacteria for organic material removal and play an important role in nitrogen and phosphorus cycling, with functions in nitrification and denitrification [12]. They are also the core bacterial community in the shrimp gut and are beneficial for shrimp health and growth [32]. Acidobacteria is dominant phyla only in samples of the PBC system and has the ability to degrade starch and cellulose, so it is the dominant phylum in the PBC system that degrades starch and cellulose, which are abundant in *C. pyrenoidosa* [33]. The results of this study showed that the decrease in the relative abundance of Proteobacteria and Bacteroidetes and the increase in the relative abundance of Acidobacteria in PBCA and PBCF resulted from the supplementation of *C. pyrenoidosa* to the PBC system. Planctomycetota can transform TAN and NO_2^-—N to nitrogen under low DO conditions through the anammox pathway [34]. Compared with IBF, the relative abundance of Planctomycetota increased with increasing bacterial community diversity in biofilms. In addition, the higher relative abundance of Planctomycetota in the biofilms may have resulted from the depletion of oxygen in the pores by the adsorbates. Chloroflexi seemed to be an important bacterium for biofilm formation and can degrade macromolecular organic compounds into low-molecular-weight substrates for its growth and the growth of other bacteria so that the relative abundance of Chloroflexi was higher in the biofilms than in the adsorbates [35]. *Pseudohongiella* and *Catenovulum* were dominant genera in the PB systems. *Pseudohongiella* can enhance the transfer of organic matter and metabolism to increase its utilization [36]. *Catenovulum*, a common marine probiotic, can degrade proteins and polysaccharides and increase the resistance of aquatic organisms to *Vibrio* [37]. *Acanthopleuribacter* and *Bowmanella* were dominant genera only in the PBC system due to the supplementation of *C. pyrenoidosa*. The relative abundance of *Acanthopleuribacter* in PBCA and PBCF was 57.5% and 24.9%, respectively, and it degraded residues of *C. pyrenoidosa* adsorbed in the pores of polyurethane sponges [38]. Moreover, *Bowmanella* had an alga-lysing effect and improved the digestion and absorption of algae nutrients [39]. The results of this study showed that the relative abundance of nitrifying bacteria in PBCF and PBOF decreased from 10.18% to 3.05% and 0.75%, respectively. Del'-Duca et al. [40] reported that the nitrification capacity does not decrease, although the relative abundance of nitrifying bacteria decreased from 30% to less than 3% with the maturity of biofilms. *Nitrosococcus*, *Nitrosomonas*, and *Halomonas*, which belong to Proteobacteria, were the dominant genera in IBF, and all carried out the function of ammoxidation [41]. However, the relative abundances of *Nitrosococcus* and *Halomonas* in the PB systems were less than 0.01%. The relative abundances of *Nitrosomonas* in PBCF and PBOF were 0.56% and

0.45%, respectively, and it played an important role in the oxidation of ammonia to nitrite in this study. *Nitrospira* is the only genus detected in Nitrospirota in this study, which has the function of nitrite oxidation and comammox [41]. In contrast to the decrease in the relative abundance of *Nitrosococcus* and *Nitrosomonas*, the relative abundance of *Nitrospira* in PBCF and PBOF increased from less than 0.01% to 2.36% and 0.07%, respectively, because *Nitrospira* could not proliferate in an environment where the energy sources were only reduced inorganic nitrogen. The results also showed that the relative abundance of *Nitrospira* was higher in systems with higher concentrations of toxic nitrogen. *Nitrococcus*, which belongs to Proteobacteria, plays an important role in the oxidation of nitrite to nitrate. *SM1A02* was the dominant genus in biofilms and participated in multiple nitrogen cycling processes, including nitrification, denitrification, and anammox, due to the thickness of biofilms and a variety of bacterial symbioses [42, 43]. The dominant genus with the denitrification function in IBF was *Hyphomicrobium*, while those in the PB systems were *Ruegeria*, *Rheinheimera*, and unclassified_f__Rhodobacteraceae. The results showed that of the systems, the PBO systems had the highest relative abundance of denitrifying bacteria, which decreased as the relative abundance of *Acanthopleuribacter* increased. In short, the results of this study indicate that *C. pyrenoidosa* has practical application prospects in aquaculture systems of *L. vannamei* because the supplementation of *C. pyrenoidosa* has a positive effect on shrimp growth.

5. Conclusions

In the present study, the effects of *C. pyrenoidosa* on the water quality and growth performance of *L. vannamei* in PB and WE systems were evaluated. Supplementation with *C. pyrenoidosa* increased turbidity and the concentrations of TAN and NO_2^-—N in the PB systems but had no significant effects on water quality parameters in the WE systems. Moreover, the final concentrations of TP in the PBC and WEC systems decreased by 15.74% and 23.33%, respectively. With the supplementation of *C. pyrenoidosa* to the systems of PBC and WEC, the final weight of *L. vannamei* increased by 19% and 16%, respectively. With the supplementation of *C. pyrenoidosa* to the PBC system, the relative abundances of Proteobacteria and Bacteroidetes decreased, while the relative abundance of Acidobacteria increased in PBCA and PBCF. The relative abundances of *Acanthopleuribacter*, *Bowmanella*, and *Nitrospira* in the PBC system were significantly higher than those in the PBO system.

Data Availability

All data generated or analyzed during this study are included in this published article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Yongxin Liu has contributed to the conceptualization, methodology, project administration, writing—the original draft, writing—review and editing, and supervision. Ailing Xu has contributed to the methodology, formal analysis, investigation, and data curation. Chao Liu has contributed to the methodology, data curation, and investigation. Mingzhu Sun has contributed to the conceptualization, methodology, project administration, investigation, data curation, and writing—the original draft. Zhiwen Song has contributed to the conceptualization, methodology, funding acquisition, supervision, writing—review and editing, and project administration.

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