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Rapid Production Biofloc by Inoculating *Chlorella pyrenoidosa* in a Separate Way

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Abstract: Microalgae play an important role in the formation of biofloc. To demonstrate the feasibility of *Chlorella pyrenoidosa* in biofloc formation, an experiment was performed with a simple random design consisting of five inoculation levels (in triplicate) of *C. pyrenoidosa* (0, 1×10^8, 1×10^9, 5×10^9, and 1×10^{10} cells·L^{-1}) in the biofloc system. All treatments kept a C:N ratio of approximately 15:1. This study observed the effects of different initial concentrations of *C. pyrenoidosa* on biofloc formation, water quality and bacterial community in biofloc systems. The results indicated that *C. pyrenoidosa* had the ability to enhance biofloc development, especially when the *C. pyrenoidosa* initial concentration reached 5~10 $\times$ 10^9 cells·L^{-1}. Too high or too low a concentration of *C. pyrenoidosa* will adversely affect the formation of biofloc. The effect of *C. pyrenoidosa* addition on water quality (TAN, NO_2^--N, and NO_3^--N) was not significant in the final stage. The inoculation of *C. pyrenoidosa* decreased the species richness and diversity of the bacterial community but increased the domination of *Proteobacteria* and *Bacteroidota* in the biofloc system, especially the order of *Rhizobiales*. The addition of *C. pyrenoidosa* could maintain water quality by increasing the proportion of several denitrifying bacteria, including *Flavobacterium*, *Chryseobacterium*, *Pseudomonas*, *Brevundimonas*, *Xanthobacter*, etc. These above dominant denitrifying bacteria in the biofloc system could play a major role in reducing the concentration of NO_2^--N and NO_3^--N. So, we recommended the reasonable concentration is 5~10 $\times$ 10^9 cells·L^{-1} if *C. pyrenoidosa* is used to rapidly produce biofloc.

Keywords: *Chlorella pyrenoidosa*; biofloc formation; water quality; bacterial community

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1. Introduction

The aquaculture industry plays an important role in providing aquatic products for the world population through intensive cultivation. However, as aquaculture cultivation intensifies, researchers found that only 20–30% of nitrogen is utilized and retained in organisms [1]. That is, around 70–80% of nitrogen is released into the adjacent environment, which could produce an adverse effect [2,3]. Biofloc technology (BFT) was considered to play an important role in maintaining the rearing water quality and providing extra nutrition for aquaculture animals [4,5]. This technology could solve the adverse effect from nitrogen discharge by constructing biofloc systems [6,7]. This system is generally constructed by providing organic carbon and maintaining the C/N ratio at a reasonable level to remove toxic nitrogen [8,9]. As a microbial-based system, its structure is considered to contain 29% microalgae, 35% bacteria, 24% fungi and 12% zooplankton [10]. Microalgae, as one of the main components, is involved in the formation of biofloc. For instance, *Chlorophyceae*, *Bacillariophyceae*, and *Cyanophyceae* were discovered as the most common species in the initial stages of the biofloc system [11,12]. In addition, *Chlorella*, *Acutodesmus* and *Chlamydomonas* have been regularly found to be a stable biofloc composition [13]. These microalgae not only participate in the formation but also play a key role in removing nitrates, providing oxygen to heterotrophic bacteria and enhancing the aggregation

of microorganisms in the water by their excreta [14–16]. Many studies thought that the enhancing effect of microalgae is important in the formation of biofloc. Loria et al. [17] found that the addition of *Chlorella vulgaris*, *Chlorella sorokiniana*, *Scenedesmus dimorphus*, and *Neochloris oleoabundans* could form biofloc. The addition of *Chlorella* sp., *Grammatophora* sp., and *Navicula* sp. could increase the quantity of protein and lipids in biofloc [17,18]. It was proven that the improvement of *C. vulgaris* on floc was caused by secreting extracellular metabolites, which could provide a high level of flocculating activities [19,20]. This suggested that the choice of appropriate species could be a key factor that influenced the effect of biofloc formation. However, these studies were conducted using conventional production, culturing biofloc in the same units with farmed animals [21]. To focus on the quality of the biofloc and facilitate the quantification of biofloc production, the biofloc needs to be cultured in a separate way [22,23].

Compared with *C. vulgaris*, *Chlorella pyrenoidosa* (*C. pyrenoidosa*) was found to produce a higher biomass [24]. It is a widely distributed and cultivated freshwater microalga that is extensively used in aquaculture as food for aquaculture animals [25]. According to the Food and Agriculture Organization (FAO), *C. pyrenoidosa* is one of the healthy green foods, rich in nutrients such as protein, total lipids and total carbohydrates [26]. In addition, *C. pyrenoidosa* is characterized by a high reproduction rate and the ability to use carbon sources for mixotrophic growth [27,28], especially the strong ability to convert ammonia to high nutritional biomass under a mixotrophic mode [29]. This evidence indicated that *C. pyrenoidosa* was regarded as the most potentially promising species for producing biofloc. However, the effects of *C. pyrenoidosa* on the formation of biofloc are still unknown. So, we explored the effects of different initial concentrations of *C. pyrenoidosa* on the formation amount, water quality and bacterial community under the novel separate production of biofloc. The aim of this study was to obtain which initial concentration of *C. pyrenoidosa* would be suitable for the formation of biofloc. We tried to reveal the promotion mechanism of *C. pyrenoidosa* on biofloc through water quality and bacterial community. This is vital in providing some fundamentals for elaborating the rapid establishment of biofloc by inoculating *C. pyrenoidosa*.

2. Materials and Methods

2.1. Microalgae and Bacteria Strain

C. pyrenoidosa (FACHB-9) was obtained from the Freshwater Algae Culture Collection at the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China). This microalga was grown in BG11 medium sterilized at 121 °C for 20 min. The cultivation was performed at 25 ± 1 °C with a 12:12 light-dark cycle. In the study of biofloc formation, *Bacillus subtilis*, as a kind of probiotic bacterium in aquaculture, was widely applied to improve the formation process [30,31]. So, we selected this bacterium as an inoculating strain for our biofloc system under separate production. *B. subtilis* was obtained from Baiwo co., Ltd. (Beijing, China) and was activated by inoculating it into ultra-water under sterile conditions and culturing it at 37 °C for 24 h with agitation.

2.2. Experiments Design

The experimental system consisted of a reactor (measuring cup, 3 L), a cylindrical aeration stone and an air pump. Five treatments were set up for the current experiments, including different inoculation concentrations of *C. pyrenoidosa* (0, 1×10^8, 1×10^9, 5×10^9 and 1×10^{10} cells·mL^{-1}) with three replications (Table 1). *B. subtilis* was added to each reactor to a final concentration of 2×10^7 CFU·mL^{-1}, which was adjusted on the basis of our pre-experiments based on the results of Yusufi et al. [32]. All treatments were run at 25 ± 1 °C and in a 12:12 light-dark cycle for 13 days. Glucose was used in all treatments as a carbon source, while urea was used as a nitrogen source. The ratio of carbon and nitrogen (15:1) was maintained through the additional carbon and nitrogen source. No water exchange was carried out in the biofloc treatments; however, the regular addition of freshwater was performed to replace water loss due to evaporation. During a 13-day ex-

perimental period, 100 mL of water in biofloc reactors was collected and filtered through a 0.45 μm pore size membrane under vacuum pressure through a pump. Then, filter papers were used to determine the total suspended solid (TSS) [33], and the filtrate was used to analyze the water quality.

Table 1. *C. pyrenoidosa* initial inoculation concentrations of the treatments in experiment.

Treatments	A	B	C	D	E
C. pyrenoidosa (cells·L^{-1})	1×10^8	1×10^9	5×10^9	1×10^{10}	0
B. subtilis (CFU·mL^{-1})	2×10^7	2×10^7	2×10^7	2×10^7	2×10^7
Volume (L)	3	3	3	3	3
C/N	15	15	15	15	15

2.3. Analytical Methods

2.3.1. Biofloc Development Analysis

Changes in TSS concentrations and turbidity over time can indicate the biofloc development in water [34]. To determine TSS, an unused filter of 0.45 μm pore size was dried in a dryer at 105 °C and then weighed on an electronic microbalance. The filter was dried after filtering a 100 mL biofloc water sample. The net dry weight was calculated by subtracting the weight of the unused filter from the final weight [35]. The turbidity was tested with the turbidity meter (430IR, Turb, MUC, GER).

2.3.2. Water Quality Analysis

Water quality parameters, including total ammonia nitrogen (TAN), nitrite nitrogen (NO_2^--N), nitrate nitrogen (NO_3^--N), temperature (T), pH and dissolved oxygen (DO), were measured once every two days. The concentration of TAN, NO_2^--N, and NO_3^--N were analyzed using a flow-injection analyzer (QC8500, Hach, Loveland, CO, USA). The physicochemical variables of T (°C), pH, and DO (mg·L^{-1}) were measured in situ using the multiparameter (ProQuatro, YSI, Yellow Springs, OH, USA).

2.3.3. Bacterial Community Analysis

To investigate the impact of *C. pyrenoidosa* on bacterial community composition more comprehensively, three time points were chosen to analyze the bacterial community. Water samples were filtered through a 0.22 μm pore size membrane and stored frozen at −80 °C for further analysis. These samples were sent to Yuanxin Co. (Shanghai, China) for the sequencing of the 16S rDNA V4-V5 region and biofloc bacterial diversity analysis (primer sequence information, 515F: 5′-GTGCCAGCMGCCGCGG-3′ and 907R: 5′-CGGTCAATTCMTTTRAGTTT-3′) [5]. Operational taxonomic units (OTUs) were clustered with a 97% similarity cutoff using UPARSE (version 7.0.1090). The bacterial diversity was measured using Shannon and Simpson indexes, while the Chao1 and Ace indexes were used to reflect species richness. These four parameters were determined based on the calculated OTUs and calculated in Qiime (Version 1.9.0).

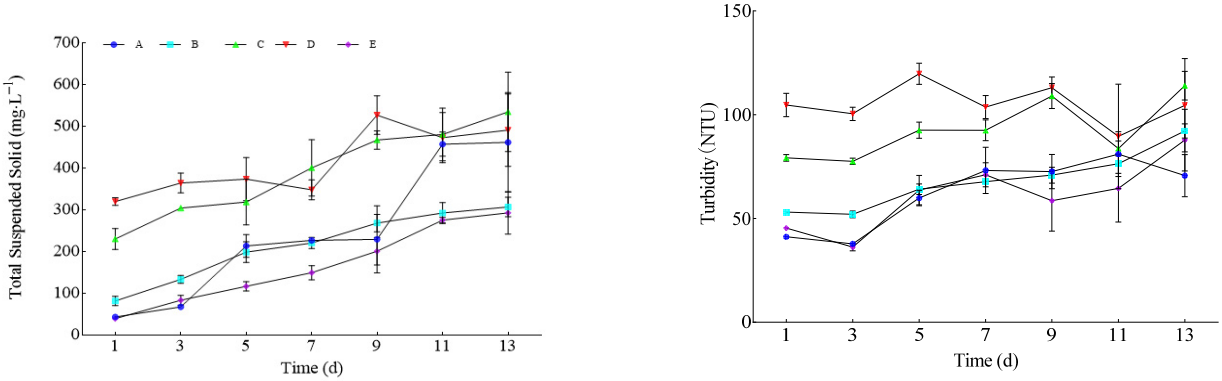
2.4. Statistical Analysis

Data were shown as the average value ± standard deviation. SPSS (Version 25.0) was used to process the data. One-way analysis of variance (ANOVA) was used to examine significant differences among the treatments of samples. Differences were considered significant when $p < 0.05$. The Duncan test was adopted for post hoc multiple comparisons if there was a significant difference among data. CCA (canonical correspondence analysis) was performed with the top 10 dominants at the genus level to clarify the relationship between environmental factor dynamics and bacterial community variations.

3. Results

3.1. Biofloc Formation Differences under Different C. pyrenoidosa Concentration

The biofloc development over time is shown in Figure 1. Both TSS (Figure 1a) and turbidity (Figure 1b) levels increased gradually throughout the experiment period. There were significant differences between the experimental treatments and the control treatment. The TSS and turbidity no longer changed significantly after the 11th day ($p > 0.05$). At the end of the experiments, the maximum TSS and turbidity were observed in treatment C, reaching about 535.02 ± 95.52 mg·L^{-1} and 110.03 ± 13.80 NTU, respectively. The least TSS and turbidity were reported with the control treatment, recording 293.00 ± 50.91 mg L^{-1} and 84.67 ± 11.82 NTU, respectively. Following these results, *C. pyrenoidosa* had the ability to enhance biofloc development. The promotional effect was the most obvious when the *C. pyrenoidosa* initial concentration reached 5~10 × 10^9 cells·L^{-1}.



(**a**) (**b**)

Figure 1. The variation in (**a**) total suspended solids (TSS) levels and (**b**) turbidity of five treatments.

3.2. Change in Water Quality under Different C. pyrenoidosa Concentration

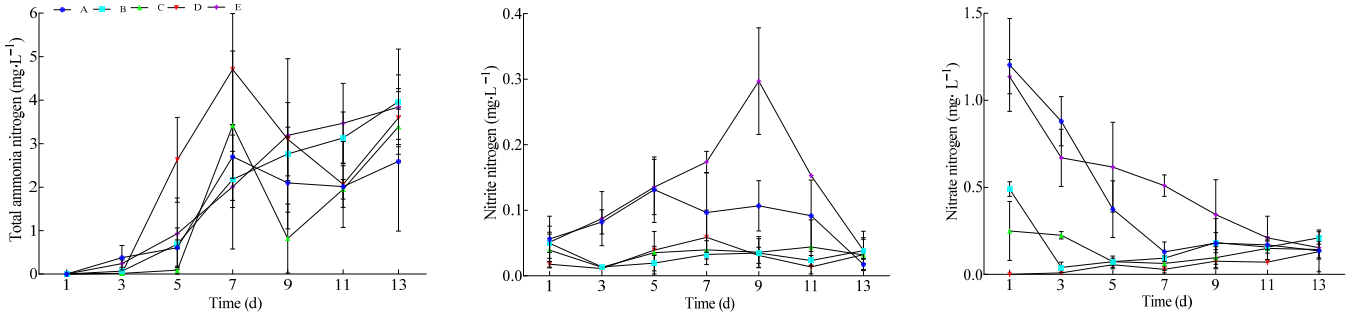
The water quality parameters of experimental and control treatment are shown in Table 2. The water temperature, pH and DO in all treatments remained stable and were within the ideal range throughout the trial.

Table 2. Water quality parameters in five treatments throughout the 13-day experimental period.

Treatments	pH	DO (mg·L^{-1})	Temperature (°C)
A	8.38 ± 0.35	5.84 ± 1.37	25.19 ± 0.32
B	8.55 ± 0.30	6.46 ± 0.94	25.28 ± 0.24
C	8.46 ± 0.42	6.77 ± 1.14	25.34 ± 0.20
D	8.56 ± 0.37	6.25 ± 0.78	25.32 ± 0.39
E	8.37 ± 0.34	5.55 ± 1.13	24.32 ± 0.49

Note: Each value represents mean ± SD.

The concentration of TAN, NO_2^--N and NO_3^--N in five treatments during the 13-day experimental period are given in Figure 2. The TAN (Figure 2a) was not detected on day 1. The TAN was gradually increased and then maintained a relatively stable state throughout the experiment. There was no significant difference in the TAN concentration among treatments in the late stage of the culture ($p < 0.05$). The NO_2^--N (Figure 2b), showing no significant difference ($p < 0.05$) among treatments in the initial and late stages, fluctuated within a relatively low level during the middle stage. High values for NO_3^--N were seen at the start of the culture (Figure 2c). The NO_3^--N was gradually decreased and then became stagnant throughout the 13-day trial in treatments A, B, C and E. In contrast, treatment D showed accumulation during the experiment.



(**a**) (**b**) (**c**)

Figure 2. The variation in (**a**) total ammonia nitrogen (TAN), (**b**) nitrite nitrogen (NO_2^--N) and (**c**) nitrate nitrogen (NO_3^--N) of five treatments.

3.3. Dynamic Structure of the Bacterial Community under Different C. pyrenoidosa Concentration

The species richness and diversity of the bacterial community are shown in Table 3. The Chao1 and Ace indexes gradually increased over time in all treatments, while the control treatment had a higher species richness than the experimental treatments. The Simpson and Shannon indices in the experimental treatments were lower than the control treatment at the same sampling time. In summary, the inoculation of *C. pyrenoidosa* decreased the species richness and diversity of bacterial community in the biofloc system.

Table 3. The richness and diversity of the bacterial community from all treatments at different sampling times.

Time	Sample	OTUs	Richness		Diversity	
			Chao 1	Ace	Shannon	Simpson
1d	A	1006.67 ± 52.54	1405.67 ± 69.37	1871.00 ± 87.68	2.89 ± 0.17	0.12 ± 0.01
	B	1421.00 ± 48.08	1988.50 ± 86.97	2084.00 ± 43.84	3.18 ± 0.29	0.12 ± 0.01
	C	1272.00 ± 59.40	1662.67 ± 118.37	1905.00 ± 37.64	3.35 ± 0.15	0.09 ± 0.01
	D	1019.50 ± 96.87	1796.00 ± 100.41	1922.00 ± 179.61	3.48 ± 0.20	0.08 ± 0.01
	E	2556.00 ± 115.97	3651.00 ± 224.86	3531.67 ± 364.53	3.64 ± 0.18	0.10 ± 0.01
7d	A	990.00 ± 123.04	1592.00 ± 96.17	1697.50 ± 164.76	3.55 ± 0.04	0.09 ± 0.01
	B	1441.67 ± 94.73	2007.67 ± 109.04	2158.67 ± 130.60	3.33 ± 0.31	0.17 ± 0.01
	C	1350.67 ± 36.46	1947.33 ± 79.12	2508.67 ± 76.87	3.64 ± 0.24	0.06 ± 0.01
	D	1297.50 ± 36.06	2035.00 ± 104.65	2745.67 ± 242.84	3.63 ± 0.24	0.08 ± 0.00
	E	3290.67 ± 240.78	4200.33 ± 107.30	4350.00 ± 120.39	4.77 ± 0.19	0.06 ± 0.00
13d	A	1543.00 ± 183.85	2040.50 ± 293.45	2207.50 ± 300.52	3.23 ± 0.31	0.19 ± 0.01
	B	1466.33 ± 77.91	1956.33 ± 87.81	2143.67 ± 90.18	3.30 ± 0.29	0.16 ± 0.01
	C	1716.00 ± 50.91	2336.67 ± 203.01	2504.33 ± 208.71	4.10 ± 0.21	0.07 ± 0.01
	D	2385.00 ± 168.29	3114.00 ± 290.56	3759.67 ± 370.09	3.85 ± 0.21	0.10 ± 0.00
	E	3626.00 ± 113.37	4568.67 ± 37.45	4700.33 ± 66.91	5.32 ± 0.31	0.04 ± 0.01

The results of the bacterial community structure are given in Figure 3. At the phylum level (Figure 3a), A, B, C, D and E mostly consisted of *Proteobacteria* (62%, 70%, 55%, 54% and 52%), *Bacteroidota* (26%, 22%, 25%, 29% and 24%) and *Firmicutes* (5%, 1%, 2%, 1% and 6%). At the order level (Figure 3b), A, B, C, D and E mainly consisted of *Flavobacteriales* (17%, 14%, 20%, 20% and 14%), *Rhizobiales* (5%, 5%, 17%, 25% and 7%), *Caulobacterales* (11%, 27%, 8% 5% and 8%), *Pseudomonadales* (10%, 6%, 4%, 3% and 13%) and *Sphingomonadales* (6%, 8%, 7%, 7% and 1%). The predominant genus is shown in Figure 3c. There were many unidentified species which illustrated the complexity of bacterial communities in biofloc systems. A, B, C, D and E mostly consisted of *Flavobacterium* (14%, 9%, 17%, 20% and 15%),

Brevundimonas (11%, 32%, 9%, 5% and 2%), *Chryseobacterium* (8%, 9%, 12%, 11% and 4%), *Pseudomonas* (11%, 6%, 5%, 4% and 5%) and *Xanthobacter* (3%, 3%, 10%, 10% and 7%).

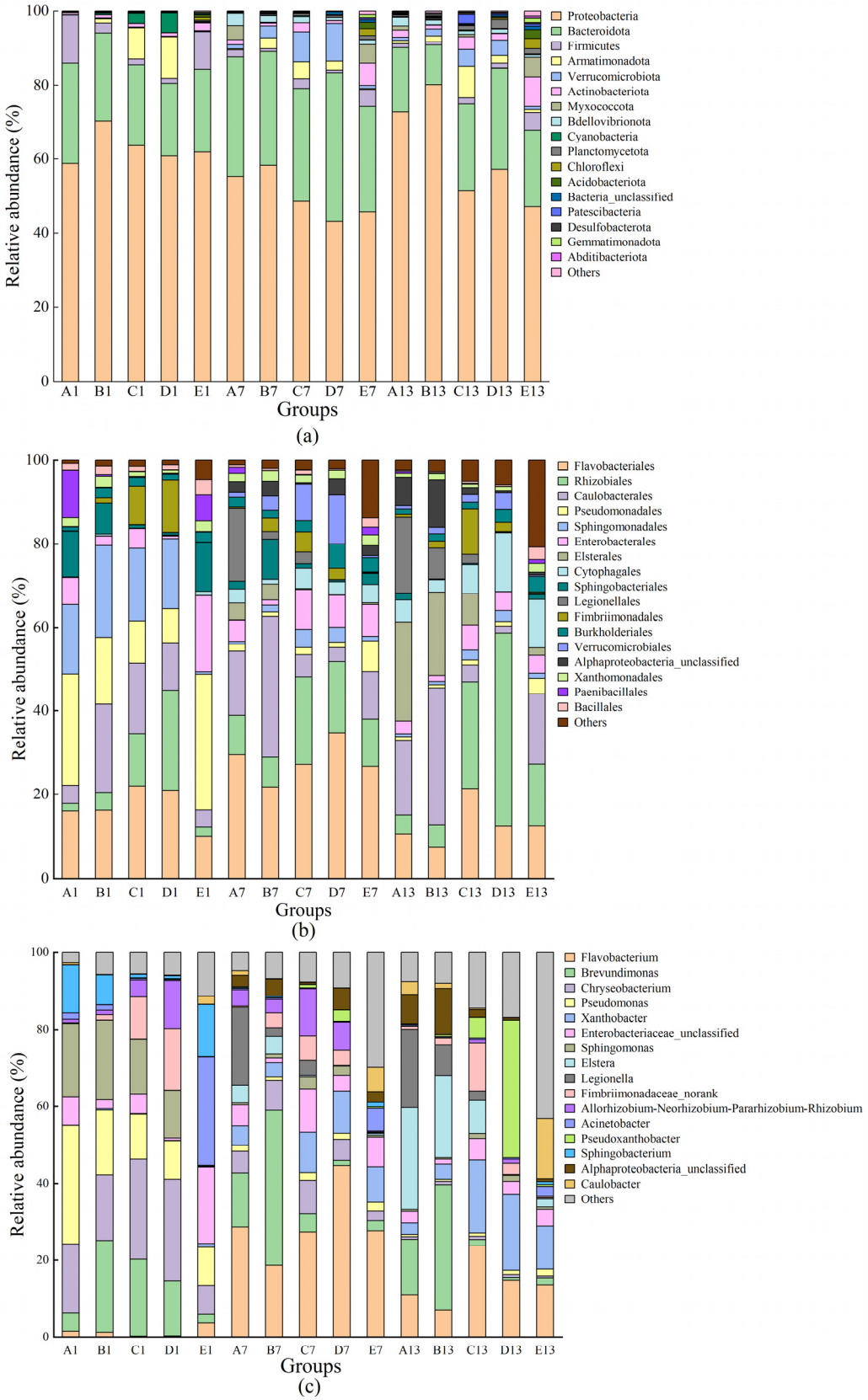


Figure 3. Bacterial community at (**a**) phylum, (**b**) order and (**c**) genus levels of five treatments.

The result of CCA is shown in Figure 4. A positive correlation existed between TSS, turbidity and TAN, whereas a negative correlation existed between these variables and NO_2^--N and NO_3^--N. *Xanthobacter*, *Flavobacterium* and *Brevundimonas* were influenced by TSS and turbidity. NO_2^--N had a positive relationship with *Fimbriimonadaceae-norank*. The dominant bacteria that were positively associated with NO_3^--N included *Sphingomonas*, *Chryseobacterium*, *Pseudomonas* and *Enterobacteriaceae-unclassified*. *Elstera* and *Legionella* were influenced by TAN.

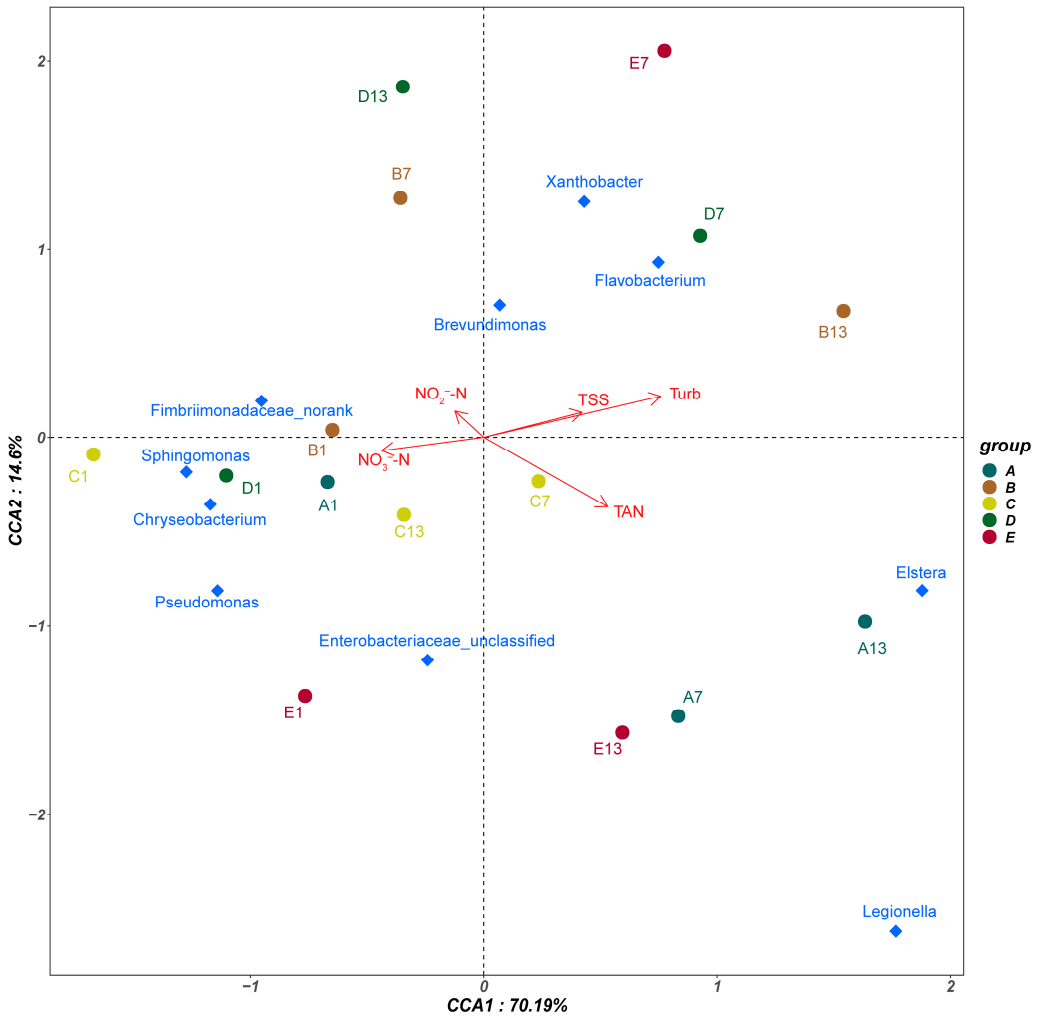


Figure 4. Canonical correspondence analysis (CCA) with water parameters and bacterial community (top 10 dominant at genus level) in biofloc system. TAN, total ammonia nitrogen; NO_2^--N, nitrite nitrogen; NO_3^--N, nitrate nitrogen; TSS, total suspended solid; Turb, turbidity. The water parameters were indicated as arrows. The dominant bacteria were represented as lozenges. Samples were indicated as circles, with numbers corresponding to their sampling time.

4. Discussion

4.1. Influence of C. pyrenoidosa on Biofloc Development

When the concentration of phytoplankton in the biofloc system increases, the amount of biofloc formation increases [36]. This statement is confirmed by the results that show the *Chlorella* sp. inoculum enhances the stability of biofloc formation and makes biofloc production higher [19]. So, our results indicated that *C. pyrenoidosa* could promote biofloc formation. It is very reasonable to show similar characteristics for *C. pyrenoidosa*, which is considered one of the important microalgae of *Chlorella* sp. From a macro perspective, we also found that it had no significant effect on the biofloc formation when the initial addition of *C. pyrenoidosa* was below 1×10^8 cells·L^{-1}. This finding is consistent with the result

of previous research [37], which might be attributed to the lack of influence of the lower concentration of *Chlorella* sp. (<1×10^9 cells·L^{-1}) on TSS concentration [38]. However, this microalga still can affect the particle size of biofloc from a microscopic perspective [38]. When the initial addition of *C. pyrenoidosa* exceeded 1×10^8 cells·L^{-1}, it could significantly promote the biofloc formation. Nevertheless, the biofloc system with *C. pyrenoidosa* addition (1×10^{10} cells·L^{-1}) demonstrated lower production during the final phase. This may have been due to the fact that a high TSS concentration reduces the light entrances into the system, intervenes in the growth of *C. pyrenoidosa* [39] and inhibits the growth of other phytoplankton [40].

4.2. Influence of C. pyrenoidosa on Water Quality

In the present study, the addition of *C. pyrenoidosa* did not affect TAN, NO_2^--N and NO_3^--N. The same evidence was revealed in the *Chlorella* sp. addition, which did not significantly influence TAN and NO_2^--N [41]. Although *Chlorella* sp. generally has the ability to utilize carbon sources for growth to remove or uptake TAN [42], it does not have enough ability to transform TAN through the production of bacterial biomass for a large amount of glucose added [43]. Thus, it could have been caused by homologous reasons as *C. pyrenoidosa* is one of the dominant microalgae of *Chlorella* sp. Additionally, TAN showed an increasing trend until the end of all treatments, which was mainly due to the degradation of dead biomass [23] and the development of denitrifying bacteria during biofloc formation. Contrary to TAN changes, NO_3^--N went down in four treatments except treatment D, which could have happened due to the following three mechanisms: aerobic heterotrophic denitrification into nitrogen gas [19], dissimilatory nitrate reduction to ammonium (DNRA) [44] and heterotrophic assimilated into bacterial biomass [45].

4.3. Influence of C. pyrenoidosa on Bacterial Community

The observation that microalgae reduced bacterial richness and diversity in biofloc systems has also been reported [46]. We further formalized the above view in our research about the addition of *C. pyrenoidosa*. However, the dominant bacteria were indeed selective in the biofloc formation. In this condition of high carbon, *Proteobacteria* could produce a variety of metabolic species that could degrade organic matter as well as remove nutrients [47]. We have found that *Pseudomonadales*, *Caulobacterales* and *Sphingomonadales* belonging to the phylum of *Proteobacteria* were the four main orders playing a part in the above metabolic process. In particular, the order of *Rhizobiales* was proved to be conducive to the nutrient cycling and organic compounds' utilization in biofloc systems [48,49]. In addition, the order of *Flavobacteriales* belonging to the phylum of *Bacteroidota* also has a high capacity to use organic compounds [50]. A similar phenomenon has been verified in other studies [5,51]. All of this could reasonably explain why the two bacteria (*Proteobacteria* and *Bacteroidota*) at the phylum level were dominant in our research and why the relative abundance of *Rhizobiales* at the order level increased with the increasing concentration of *C. pyrenoidosa*. Meanwhile, several denitrifying bacteria were considered as the main bacteria in the heterotrophic and aerobic conditions designed in this study. Under this circumstance, as the members of *Bacteroidota*, *Flavobacteriales* (*Flavobacterium*) were not only associated with heterotrophic denitrification [52] and the accumulation of nitrogen [53] but also had the ability to increase the flocculation efficiency of several green algae cultures [54]. In addition, *Chryseobacterium* was considered to be capable of utilizing NO_3^--N aerobically in the presence of NH_4^+-N [55]. We also found that *Pseudomonas*, *Brevundimonas* and *Xanthobacter* were the dominant genera in the phylum of *Proteobacteria*. *Pseudomonadales* (*Pseudomonas*) had the characteristics of heterotrophic nitrification and aerobic denitrification simultaneously [56], while *Brevundimonas* could perform aerobic denitrification with high nitrate [57,58]. *Xanthobacter* also have the ability to make the dissimilatory reduction of both NO_3^--N and NO_2^--N to gaseous forms of nitrogen [59]. This evidence could also reasonably explain why these above bacteria at the genus level were dominant in our research.

5. Conclusions

The present research clearly demonstrated that the addition of *C. pyrenoidosa* could promote the production efficiency of the biofloc. Especially when the initial concentrations of *C. pyrenoidosa* were in a reasonable range of 5~10 $\times$ 10^9 cells·L^{-1}, it was the better-inoculated level for the rapid formation and stability of the biofloc. Too low a concentration could not produce a macroscopically visible effect, while too high a concentration would inhibit the performance of the biofloc production. It is an important point that needs to be taken into account for the higher TSS concentration, which could reduce the light entrances into the system and intervene in the photosynthesis and the growth of microalgae. We also proved that the addition of *C. pyrenoidosa* could bring about the reduction in species richness and diversity. At the same time, the inoculation of this microalgae could make *Proteobacteria* and *Bacteroidota* dominant in the biofloc system, while the order of *Rhizobiales* could be a main biological factor promoting biofloc formation. In the process of biofloc formation, the concentration of TAN over time was raised under the addition of *C. pyrenoidosa*, which could be ascribed to the existence of three functions: the transformation of TAN, the degradation of dead biomass, and denitrification in the biofloc system. CCA analysis proved that the amount of biofloc formation was negative with NO_2^--N and NO_3^--N, as several dominant denitrifying bacteria in the biofloc system played a major role in the formation. Furthermore, the inoculation of *C. pyrenoidosa* could maintain water quality by increasing the proportion of several denitrifying bacteria (such as *Flavobacterium*, *Chryseobacterium, Pseudomonas*, *Brevundimonas*, *Xanthobacter*, etc.).

Author Contributions: All authors listed have contributed to this study. Conceptualization, R.Z. and J.Z.; methodology, Y.C., Z.F. and Z.S.; software, Y.C. and R.Z.; validation, R.Z., J.Z., Q.X. and Y.Z.; formal analysis, J.Z., Q.X. and Y.Z.; investigation, R.Z.; resources, R.Z. and Y.Z.; data curation, Z.F. and Z.S.; writing—original draft preparation, Y.C.; writing—review and editing, Y.C., R.Z. and Y.Z. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data that support the findings of this research are available from the corresponding author (Zhang, R.), upon reasonable request.

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Conflicts of Interest: The authors declare no conflict of interest.

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