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Study on the water-saving and pollution-reducing effect of biofilm–biofloc technique in *Anguilla marmorata* aquaculture

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ABSTRACT

Daily water exchange rate was over 50% in the traditional intensive culture for *Anguilla marmorata*, which resulted in a huge waste of fresh water resources and the pollution of surrounding water. To save water and reduce pollution, the comparison experiment on biofilm–biofloc technique applied in *Anguilla marmorata* intensive culture was implemented. Nine tanks were randomly divided into three groups. Biofilm water-cleaning grille was set up at 7.1% of water volume, and supplementary sucrose was added to water at 75% of feed per day in treatment group I; biofilm water-cleaning grille was set up at 7.1% of water volume, and supplementary starch was added to water at 75% of feed per day in treatment group II; the other group III without any treatment as the control group. The results showed that the daily water exchange rate of treatment group I and treatment group II were significantly lower than the control by 69.2% and 74.4%, respectively ($p < 0.05$). The concentrations of total ammonia nitrogen, nitrite nitrogen, nitrate nitrogen, total nitrogen, total phosphorus, solved reactive phosphorus (SRP) and *Vibrio* density in treatment group I were lower than the control by 43.5%, 38.3%, 32.4%, 22.4%, 35.8%, 32.9%, and 45%, respectively ($p < 0.05$). While, the concentrations of the same mentioned parameters except SRP in treatment group II were lower than the control by 27.2%, 51.7%, 37.8%, 33.3%, 20.4%, and 50%, respectively ($p < 0.05$). The growth rate of treatment group I and treatment group II were significantly higher than the control by 37.8% and 14.9%, respectively ($p < 0.05$). Therefore, biofilm–biofloc technique had remarkable water-saving and pollution-reducing effect and should be extensively used in aquaculture.

Keywords: Carbon source supplements; Total ammonia nitrogen; *Vibrio*; Water exchange rate; Water treatment

1. Introduction

Anguilla marmorata (the giant mottled eel) belongs to Anguilliformes, Anguillidae, *Anguilla*. The flesh has advantages of high protein content and low fat content, and is rich in essential amino acids, unsaturated fatty acids and mineral elements. The nutritional value is better than that of Japanese eel and European eel [1]. About 95% of *A. marmorata* fingerlings in China are from Philippines. The culture began in 2005, and then the aquaculture scale gradually formed [2]. The protein conversion rate of eel feed is only 27.4%, with average crude protein content of 45%, and average feed efficiency of 65%. A large amount of nitrogen is excreted in the

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form of residual feed and feces, resulting in serious water pollution and the increase of eel disease [3]. *Vibrio* sp. is one of the most important pathogen of fish [4,5]. *Vibrio* disease is one of the main bacterial disease of cultured eel, which is commonly caused by *Vibrio vulnificus* or *Vibrio anguillarum*. The disease results in body hemorrhage, dermis ulceration, hepatomegaly and hemorrhage in the liver. The mortality rate is high, and the harm is so severe that over 50% of the infected eels have died so far [6,7]. On the other hand, for Japanese eel cultured in concrete pond, the daily water exchange rate in eel fingerling period is 30%–50%, and it is 50%–100% in growing out yellow eel period, while the daily water exchange rate of European eel and American eel is up to 100%–150% [8], leading to a huge waste of freshwater and the pollution of surrounding water. The biofloc technique can regulate the ratio of carbon and nitrogen (C/N) and increase the amount of heterotrophic bacteria by artificially adding organic carbon (e.g., glucose) in water. Using microorganisms to assimilate inorganic nitrogen, nitrogen-containing compounds (ammonia nitrogen, etc.) are changed into bacterial proteins, forming biofloc which can be directly eaten by cultured species. Thus, this technique can solve detritus and feed retention problems in water in an eco-friendly way, achieve the re-use of feed and play multiple advantages, including water purification, reducing water exchange amount, saving feed, improving the survival rate of cultured species and increasing yield [9]. The biofloc technique has been successfully applied to the farming of *Litopenaeus vannamei* [10], tilapia [11], *Megalobrama amblycephala* [12], etc., and has obtained several advantages, including improving water quality and increasing feed utilization. By setting biofilm water-cleaning grille in aquaculture pond, a large number of biofilm can be formed on the surface of grilles [13]. The biofilm, which is rich in bacteria, algae, etc., can effectively decompose organic matters in water, improve water quality, and some bacteria may absorb organic debris, suspended particles, etc., to form biofloc, which is subsequently eaten by the eel, and then the re-use of feed proteins will be achieved [13]. The technique has obvious advantages, including water saving, pollution reducing, energy saving, yield increasing and income increasing, and has been applied to the cultivation of *L. vannamei*, Japanese eel, grass carp, loach, etc. [14,15]. In the present study, the advantages of biofilm and biofloc techniques were combined, and the effect of biofilm–biofloc technique in *A. marmorata* aquaculture on water saving and pollution reducing was investigated.

2. Materials and methods

2.1. Experimental materials

The experiment was carried out in nine round polyethylene buckets, each with 1.5 m^3 of water and 0.5 m of water depth. A microporous aeration tube for oxygen inflation was equipped on bucket bottom. The average body length of *A. marmorata* fingerling was 32.1 ± 2.42 cm, the average body weight was 95.1 ± 4.1 g. One hundred and fifty fingerlings were placed in each bucket, and the density was 9.5 kg/m^3. The brown sugar and wheat starch were purchased from a farmers' market. The biofilm water-cleaning grille for aquaculture (China Patent No. ZL20112003-2516.6, shown in Fig. 1), composed of environment friendly elastic stuff, was 2.0 m long and 0.7 m high after cutting, and one strip was vertically hanged in each bucket of the treatment groups. The aquaculture water was fresh tap water after aeration. Eel fingerling feed was purchased from Fujian Tianma company (Paibian industrial zone, shangjing town, fuqing city, fujian province, China), with crude protein content ≥47%.

2.2. Establishment of treatment groups and control group

The experiment was performed in the eel culture workshop of Jimei University, and the trial period was 105 d. Nine buckets were randomly assigned into three groups, three buckets each. Biofilm water-cleaning grille was set in buckets at 7.1% of water volume, and supplementary sucrose was added to water at 75% of feed weight per day in treatment group I; biofilm water-cleaning grille was set in buckets at 7.1% of water volume, and supplementary starch was added to water at 75% of feed weight per day in treatment group II; the other group III without any treatment as the control group.

2.3. Aquaculture management

The eel feed was given twice a day, respectively, at 8:00 am and 6:00 pm, and the daily feeding rate was about 1.5% of eel weight. In the treatment groups, the dosages of brown sugar or starch was weighed 1 hour after feeding every morning, and then added to culture water, respectively. In the premise of ensuring the normal growth of *A. marmorata*, the water exchange amount was minimized in each group. The amount of feed was appropriately increased with the eel growing. The amount of feeding, the amount of water exchange, the incidence and death of fish were determined daily during the trial period. Eel was weighed after the trial. The amount of feed was recorded. The survival ratio, the growth rate and the feed conversion ratio (FCR) were calculated.

2.4. Dosage of additional carbon source

The dosage of additional carbon source was determined using the following formula [16]:

$$\Delta\mathrm{CH} = \frac{\Delta\mathrm{Feed} \times \%N\ \mathrm{feed} \times \%N\ \mathrm{excretion}}{0.05}$$

In the formula, ΔCH refers to the daily dosage of carbon source (g), ΔFeed refers to the daily feeding amount (g),

Fig. 1. Biofilm water-cleaning grille for aquaculture.

%*N* feed refers to the nitrogen content of the feed (%), and %*N* excretion refers to the nitrogen excretion rate of the feed, which is about 50%. Because fish or shrimp (e.g., *Litopenaeus vannamei*) in the pond assimilate only about 25% of the nitrogen added in the feed. The rest is excreted mostly as NH_4 (some as organic *N* in feces or feed residue). It can be assumed that at least 50% of the feed nitrogen is excreted [9,17].

In this study, the calculated daily dosage of additional carbon source was about 76.8% of the daily feeding amount, and 75% of the daily feeding amount was actually given during the trial.

2.5. Sample collection and detection

The background water sample was collected 1 d before the test. During the trial, water samples were collected and biofloc volume (BFV) was detected every day; other water quality and microbiological parameters were detected every 15 d. Water samples were collected using a plexi glass collector at 25 cm below the central surface of the bucket, and all the samples were collected before 8:00 am, then were placed in a refrigerator containing ice cubes and transported to the laboratory for testing. The dissolved oxygen, water temperature and pH were detected using a HANNA (Shanghai, China) dissolved oxygen meter and a pH meter. Total ammonia nitrogen (TAN), nitrate nitrogen, nitrite nitrogen, SRP, chemical oxygen demand (COD) and total suspended solid were, respectively, detected using Nessler's reagent spectrophotometer, ultraviolet spectrophotometry, N-(1-naphthyl)-ethane diamine spectrophotometry, molybdenum antimony spectrophotometry, potassium dichromate method and gravimetric method [18]. The amount of total bacteria, heterotrophic bacteria, and *Vibrio* was determined using plate counting method [18–20]. BFV was determined by the Imhoff cone using natural sedimentation method [9].

2.6. Data processing and analysis

The statistics and plotting were conducted using Microsoft Excel 2007, and the results were expressed in the form of mean ± standard deviation (means ± SD). One-way ANOVA was carried out using SPSS 19.0, multiple comparisons were conducted using Duncan method, and $p < 0.05$ suggested as significant differences.

3. Results and discussion

3.1. Formation of biofloc

After the start of the experiment, the biofilm–biofloc in treatment groups quickly formed, and obvious pale brown floc was observed adhering on nylon padding on day 3 and day 6, respectively. Then, biofloc yield rapidly increased, reached a relatively stable state on day 20, tended to decrease after reaching a peak on day 35, and then reached a new peak on day 95. The yield of biofilm–biofloc in treatment groups was calculated by counting the biofloc weight on each nylon thread, and the unit was mg/thread. During the trial, the average biofilm–biofloc content of treatment group I and treatment group II was 66.2 ± 3.1 and 22 ± 1.2 mg/thread, respectively. In the middle-late period, there were lots of *Limnodrilus hoffmeisteri* and protozoa in the biofloc. During the trial, the biofloc content increased from the initial stage to the peak and then tended to decrease to a certain stable content. In the stable period on day 35 to 37, the biofloc content in aquaculture water of treatment group I and treatment group II was 1.36 ± 0.47 and 0.33 ± 0.121 mL/L, respectively.

3.2. Water quality parameters

At the beginning of the trial, there was no significant difference in all water quality parameter concentrations ($p > 0.05$). During the trial (water quality parameters of each group were shown in Table 1 and Figs. 2 and 3), there were no significant differences in water temperature, pH, dissolved oxygen and COD ($p > 0.05$). TAN, nitrite nitrogen, nitrate nitrogen, total nitrogen, total phosphorus, and SRP in the treatment I were significantly lower than those in the control by 43.5%, 38.3%, 32.4%, 22.4%, 35.8%, and 32.9%, respectively ($p < 0.05$). TAN, nitrite nitrogen, nitrate nitrogen, total nitrogen and total phosphorus in the treatment II were significantly lower than those in the control by 27.2%, 51.7%, 37.8%, 33.3%, and 20.4%, respectively ($p < 0.05$), while there were no significant differences in SRP ($p > 0.05$).

3.3. Microbial parameters

During the trial (microbial parameters of each group are shown in Table 2 and Fig. 4), the total bacteria in treatment

Table 1
Water quality values (means and standard deviations) in control group with C/N ratio of 6.9/1 and treatment groups with C/N ratio of 12/1 during the trial of 105 d

Water quality parameters	Control group	Treatment group I	Treatment group II
Water temperature (°C)	26.3 ± 0.24^a	26.3 ± 0.23^a	26.2 ± 0.26^a
pH	6.48 ± 0.05^a	6.47 ± 0.03^a	6.47 ± 0.10^a
Dissolved oxygen (mg/L)	7.85 ± 0.08^a	7.87 ± 0.10^a	7.98 ± 0.01^a
Nitrite nitrogen (mg/L)	0.60 ± 0.04^b	0.37 ± 0.04^a	0.29 ± 0.05^a
TAN (mg/L)	0.92 ± 0.08^b	0.52 ± 0.04^a	0.67 ± 0.05^a
Nitrate nitrogen (mg/L)	11.05 ± 0.70^b	7.47 ± 0.41^a	6.87 ± 0.28^a
Total nitrogen (mg/L)	13.51 ± 0.68^b	10.48 ± 0.23^a	9.01 ± 0.37^a
SRP (mg/L)	2.37 ± 0.21^b	1.59 ± 0.09^a	1.97 ± 0.03^{ab}
Total phosphorus (mg/L)	2.74 ± 0.14^c	1.76 ± 0.05^a	2.18 ± 0.02^b
COD (mg/L)	13.54 ± 0.78^a	13.49 ± 0.61^a	12.38 ± 0.72^a

Note: Means with different letters in the same row have significant differences ($p < 0.05$).

$^{a, b, ab, c}$: all means with a in the same row show no differences among groups ($p > 0.05$); there is significant difference between means with a and means with b or c in the same row ($p < 0.05$); there is not any significant differences between means with a and means with ab, or between means with b and means with ab ($p > 0.05$).

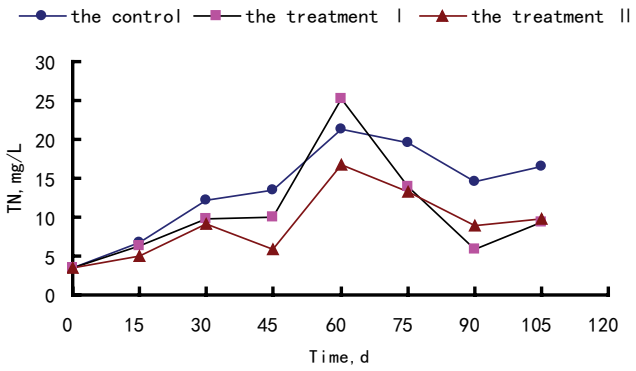

Fig. 2. Total nitrogen concentrations in the control and treatments during the trial of 105 d, total nitrogen concentrations in the treatment I and II were significantly lower than the control respectively ($p < 0.05$).

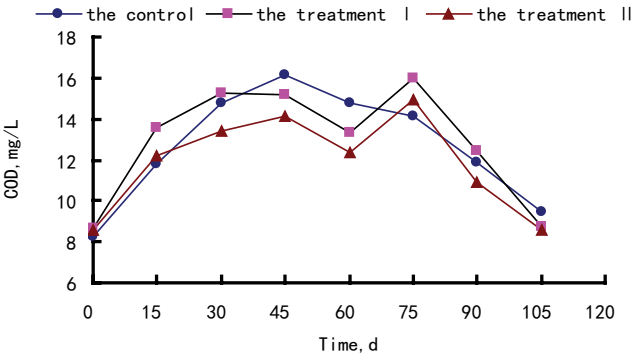

Fig. 3. Chemical oxygen demand concentrations in the control and treatments during the trial of 105 d, there were no significant differences among them ($p > 0.05$).

Table 2
Bacteria quantity in water among the control and treatments during the trial of 105 d

Microbial parameters	Control group	Treatment group I	Treatment group II
Total bacteria (10^6 CFU/mL)	1.91 ± 0.79^a	4.48 ± 1.48^b	2.09 ± 0.62^a
Heterotrophic bacteria amount (10^6 CFU/mL)	1.55 ± 0.76^a	3.52 ± 0.68^b	1.60 ± 0.73^a
Vibrio amount (10^3CFU/mL)	1.43 ± 0.17^b	0.79 ± 0.01^a	0.72 ± 0.13^a

Note: Means with different letters in the same row have significant differences ($p < 0.05$).

group I was significantly higher than that in treatment group II and control group by 114% and 134%, respectively ($p < 0.05$). Heterotrophic bacteria density in treatment group I was significantly higher than that in treatment group II and control group by 120% and 127%, respectively ($p < 0.05$). *Vibrio* density in treatment group I and treatment group

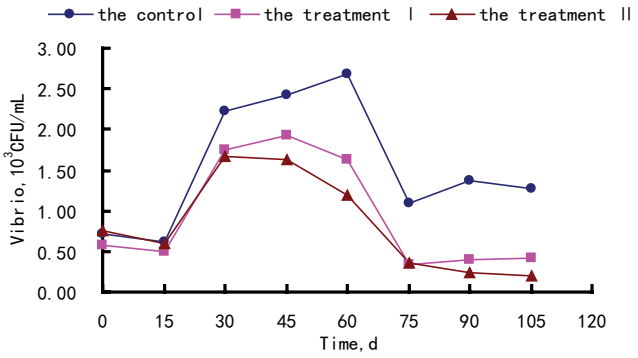

Fig. 4. Water *Vibrio* density in the control and treatments during the trial of 105 d, *Vibrio* density in the treatment I or II was significantly less than the control respectively ($p < 0.05$).

II was less than that in control group by 45% and 50%, respectively ($p < 0.05$).

3.4. Water saving and production results

As shown in Table 3, the average daily water exchange rate of the control group was 50.4%, while the daily water exchange rate of treatment group I and treatment group II was significantly lower than that of control group by 69.2% and 74.4% ($p < 0.05$). In treatment group I, the harvest individual weight and growth rate was higher than those in control group by 19.7% and 37.8%, respectively ($p < 0.05$), and the FCR was significantly lower than that in the control by 12.6% ($p < 0.05$). While in treatment group II, the harvest individual weight and growth rate were higher than those in control group by 7.9% and 14.9%, respectively ($P < 0.05$), and the FCR was lower than that in control group by 3.0% ($p < 0.05$).

4. Analysis and discussion

4.1. Formation of biofilm–biofloc.

The biofloc in aquaculture water is mainly formed by heterotrophic microorganism, combined with organic matter, protozoa, algae, filamentous bacteria, etc., by biological flocculation [21]. However, there was no algae in the biofloc in this study, for under indoor shading conditions and lack of sunlight. The crude protein content in *A. marmorata* feed was high (≥47%), while the eel could only absorb 20%–25% of the protein, and the rest was excreted in the form of total ammonia nitrogen, residual feed, feces, etc. [22], resulting in high nitrogen concentration and low C/N value in water. Therefore, it was necessary to add carbon source material to increase C/N and improve the reproduction of heterotrophic bacteria. In the case of full aeration and stirring, the suspended solids, including organic debris, could form loose biofloc with some protozoa by the flocculation. The biofilm water-cleaning grille established in treatment groups with huge specific surface area, could absorb bacteria and protozoa, which grown and formed biofilm, and then formed biofilm–biofloc by biological flocculation [13]. The formation rate of biofloc was related to carbon source

Table 3
Water saving and production results

Groups	Water exchange rate (%/d)	Harvest individual weight (g/ind)	Growth rate (g/d)	FCR
Group I	15.5 ± 0.6^a	239.3 ± 11.3^b	1.57 ± 0.15^b	1.46 ± 0.13^a
Group II	12.9 ± 2.1^a	215.8 ± 14.1^b	1.31 ± 0.15^b	1.62 ± 0.03^b
Control group	50.4 ± 2.3^b	200.0 ± 7.1^a	1.14 ± 0.03^a	1.67 ± 0.03^b

Note: Means with different letters in same column have significant differences ($p < 0.05$).

species, C/N value and culture environment, etc. When adding sucrose with 77% of the feeding amount to *L. vannamei* closed culture system, the biofloc could rapidly form on day 4 [23]. In this study, the biofilm–biofloc formed on day 3 in brown sugar treatment group, suggesting that the biofilm water-cleaning grille could benefit the flocculation of biofloc; while the biofilm–biofloc formed on day 6 in starch treatment group, which was related to the use features of heterotrophic bacteria on the two carbon sources. Brown sugar is a simple carbohydrate, easily decomposed into monosaccharides and directly absorbed by most heterotrophic bacteria; while, starch is a complex carbon compound, which cannot be directly adsorbed, and should be gradually decomposed and absorbed by some specific heterotrophic microorganism [24]. Therefore, the formation of biofloc in treatment group II was slower than in treatment group I. Adding cassava residue to *L. vannamei* culture pond, the biofloc at C/N of 10:1 formed within 6 d, and the biofloc at C/N of 15:1 formed within 4 d [25]. In this study, the C/N was adjusted to 12:1 by adding starch, and the biofloc was formed within 6 d.

4.2. Improvement of water biological factors

The total bacteria density and heterotrophic bacteria density in treatment groups were up to 10^6 CFU/mL, which was higher than that in control group; the *Vibrio* density was 10^2 CFU/mL, which was significantly lower than the control, suggesting that the biofilm–biofloc technique could increase the amount of heterotrophic bacteria and decrease the amount of *Vibrio*. *Vibrio* is one of the main pathogen of eel, the eel infected with *Vibrio vulnificus* shows skin bleeding, liver and kidney swelling, anal swelling, intestinal inflammation, etc., the mortality is high [26]. *Vibrio* grows well in water with low C/N, and when C/N ≥ 10, beneficial bacteria, including *Bacillus* and lactic acid bacteria, are greatly promoted, which inhibits *Vibrio* [27]. Bacillus secretes enzymes to degrade mucus and biofilm, and allows Bacillus and its antibiotics to penetrate the mucus layer around the Gram-negative bacteria. Furthermore, Bacillus competes for nutrients and thus inhibits other bacteria from growing rapidly. Thus any resistant bacteria cannot multiply readily and transfer resistance genes. Bacillus also competes for space on surfaces—for example, the gut wall—and displace other bacteria if they are present in high density. Because there are many different mechanisms involved in the probiotic process of competition and exclusion, it is difficult for the pathogens to resist [28]. The growth of heterotrophic bacteria can be improved by adding carbon source to increase the water C/N. The growth of heterotrophic bacteria is fast, about 10-fold of autotrophic bacteria, and it doubles every 20 min to 2 h [29]. The total bacteria and heterotrophic bacteria density in treatment group I was higher than those in treatment group II, for sucrose more likely used by heterotrophic bacteria [30]. A part of bacteria could be eaten by some protozoa, such as *Limnodrilus hoffmeisteri*, while another part could be fed by eel in the form of biofloc. Protozoa could ingest organic debris in the bottom to improve the substrate, and also used as high-quality eel feed. In addition, the biofilm water-cleaning grille established in water could provide attachment niches for autotrophic bacteria, including nitrifying bacteria, which is beneficial to promote nitrification and improve the degradation of ammonia nitrogen and nitrite nitrogen.

4.3. Improvement of water physical and chemical factors

Only 20%–25% of the protein in feed is used by aquaculture species, and the rest is present in the aquaculture environment in the form of ammonia nitrogen, residual feed, and feces [22]. Microorganisms (e.g., bacteria) in the water can decompose and use organic matters, and form ammonia nitrogen, nitrite nitrogen, and nitrate nitrogen by ammoniation, nitrification, and denitrification, resulting in serious water pollution. When ammonia nitrogen in water exceeds 1.0 mg/L, the blood binding oxygen ability of aquatic creatures will decrease, and the respiratory function will also decrease. Besides, more than 1.0 mg/L nitrite nitrogen can gradually reduce the hemoglobin count in fish, and blood oxygen-carrying capacity gradually loses, leading to hypoxia and even asphyxia death [31]. Adding starch with 30% of feed to tilapia concrete pond, the concentrations of ammonia nitrogen, nitrite nitrogen, nitrate nitrogen, total nitrogen, and total phosphorus in the treatment group were lower than those in control group [32]. In this study, the accumulation of nitrate nitrogen suggested that there were nitrifying bacteria in the treatment groups, and there were two ammonia nitrogen transformation ways in the water: heterotrophic absorption and autotrophic nitrification. Adding biofloc to shrimp culture vat could effectively decompose organic matters, and regulate the COD concentration [33]. Though there was no significant difference in COD concentrations, the water exchange amount in the treatment groups was significantly less than that in control group, and supplemental carbon source was added, suggesting that the treatment groups could effectively decrease COD. On the other hand, the increasing COD in a short time could be decreased by lots of bacteria, because the organic carbon source added in the treatment groups belonged to easy biodegraded supplements [34].

4.4. Water saving and culture effects

Based on the improvement of water biological, physical, and chemical factors in the treatment groups, the water

exchange rate of the treatment was greatly lower than the control. The growth rate and yield of the treatment were all higher than the control, when adding cassava starch as carbon source to *Penaeus monodon* culture pond [35]. Placing bamboo as ecological basis and using rice vermicelli as carbon source, the results showed that the addition of carbon source and ecological basis could significantly increase the growth rate, protein efficiency and survival rate of shrimp, and reduce the feed coefficient [36]. The biofilm water-cleaning grille and the addition of carbon source supplements in treatment groups significantly improved the growth rate of *A. marmorata*, and reduced the feed coefficient. The growth rate of *A. marmorata* in treatment group I was higher than treatment group II, and the feed coefficient was lower than treatment group II, because compared with starch, brown sugar was more favorable for the formation of biofloc, which increased the ingestion amount of the eel and the utilization rate of the feed.

5. Conclusion

The water exchange rate of treatment group I and treatment group II was significantly lower than the control by 69.2% and 74.4%, respectively ($p < 0.05$); The concentrations of TAN, nitrite nitrogen, nitrate nitrogen, total nitrogen, total phosphorus, SRP, and *Vibrio* density in treatment group I were lower than the control by 43.5%, 38.3%, 32.4%, 22.4%, 35.8%, 32.9%, and 45%, respectively ($p < 0.05$). The concentrations of the same mentioned parameters except SRP in treatment group II were lower than the control by 27.2%, 51.7%, 37.8%, 33.3%, 20.4%, and 50%, respectively ($p < 0.05$). The growth rate of treatment group I and treatment group II was significantly higher than the control by 37.8% and 14.9%, respectively ($p < 0.05$). It was suggested that using biofilm–biofloc in in-situ aquaculture water treatment, the water saving and pollution reducing advantages were remarkable, the aquaculture water quality was improved, and the growth of *A. marmorata* was greatly promoted.

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