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Research Article

The effects of rearing Pacific white-leg shrimp (*Litopenaeus vannamei* Boone, 1931) in biofloc system on the immune responses and survival rate in challenge with *Vibrio harveyi*

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Abstract

Shrimp culture, one of the most profitable industries, needs to be modified by modern techniques in Iran. The present study was focused on the effects of applied biofloc technology (BFT) on Pacific white-leg shrimp (*Litopenaeus vannamei*) culture, as a new system (zero-water exchange) to improve the immunity of shrimp and the water-quality factors in challenge with *Vibrio harveyi*. Biofloc systems were established by increasing the carbon/nitrogen (C/N) ratio by adding sugar as a carbohydrate source to the culture media. The immune system indices, and growth factors of shrimp were then measured along with water quality parameters and loads of *Vibrio harveyi* compared to the traditional water-exchange system. The results showed that using BFT led to decreasing levels of the toxic nitrogenous substances such as TAN, NO_3, and NO_2, which in turn resulted in decreasing the water exchange frequency. It was also observed that the shrimp could feed on flocs resulted in improvement the growth factor and immune system. On the other hand, despite the increased loads of *V. harveyi* bacteria, there was no significant difference in shrimp survival between the biofloc and traditional systems. These observations were confirmed by evaluating immune system factors (total hemocyte count (THC), total plasma protein and phagocytosis activity of the hemocytes. Generally, this study showed that rearing pacific white-leg shrimp in BFT in can preserve water quality and enhance shrimp's growth and immune responses, compared to the traditional systems.

Keywords: Biofloc, Shrimp, *Litopenaeus vannamei*, *Vibrio harveyi*, Immune system

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Introduction

Recently, aquaculture products have been considered as one of the most important sources for compensating the lack of protein resources in the world, especially in developing countries (Piedrahita, 2003). Controlling of diseases has been one of the essential thoughts among the farmers. They try to control nitrogenous wastes in the rearing ponds via some techniques such as polyculture of shrimp with carp species (Jewel *et al*., 2021). They also try to improve shrimp's immune-competence by supplementing diet with various immuno-stimulants such as *Spirulina platensis* (Gorgij Jaski, *et al*., 2021), sea Persian walnut (*Juglans regia*) leaves (Forouzani *et al*., 2021), cucumber meal (Javanmardi *et al*., 2020), *Gracilaria corticata* yeast (*Saccharomyces cerevisiae)* (Afsharnasab *et al*., 2016), feeding on lipid-enriched Artemia (Adloo *et al*., 2020), adjusting culture condition e.g., temperature and salinity (Yang *et al*., 2020), Carotenoprotein (Taghizadeh Andevari, *et al*., 2021), controlling the entrance of predators and contagious diseases (Soltani *et al*., 2000). Considering the problems and essential needs for increasing and expanding domestic productions in Iran, developing the high-tech industry through modern and efficient techniques is required; especially those techniques which lead to higher productions with lower costs in less area, space, water change and energy sources that reduce environmental hazards, improve health status and biosecurity (Emerenciano *et al*., 2013). In recent years, shrimp culture as one of the most profitable parts of the aquaculture industry in Iran has experienced a significant growth. Due to the high potentials for shrimp culture in Iran and the high demand in foreign countries, it could be one of the most valuable national industries (Ebne al-Torab *et al*., 2020). One of the most important problems in this industry is contaminated wastewaters from un-consumed foods and shrimp feces that led to toxic levels of ammonia and nitrite (Crab *et al*., 2007). In traditional systems, consecutive water exchanges are used to remove these contaminants from ponds. Therefore, to produce one kilogram of shrimp (Penaeidae), approximately 20 m^3 of water is required (Wang *et al*., 2008). Furthermore, the entrance possibility of the infectious diseases, especially viral agents would be increased by enhancing the water exchange rate. Also, the discharge water released into the environment would lead to serious environmental hazards (Llario *et al*., 2020). Using biofloc technology (BFT) as a modern zero-water exchange system derived from urban waste-water treatment systems could be a suitable replacement of traditional methods (Avnimelech, 2006; Llario *et al*., 2020). Biofloc means the irregular accumulation of microorganisms. In BFT, the growth and nutrition of heterotrophic and autotrophic bacteria and microalgae on aquatic waste led to remove of the waste from the water (De Schryver *et al*., 2008). Moreover, the formed biofloc could be a great source of protein for growing fish and shrimps

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(Azim and Little, 2008; Crab *et al*., 2009). Biofloc is produced through the culture systems by increasing the ratio of carbon to nitrogen and the carbohydrate source (Avnimelech, 1999; Crab *et al*., 2007). In this method, the growth and production rate of microbial mass is 10 times more than nitrifying bacteria which resulted in reducing levels of ammonia much faster than the nitrification method (Hargreaves, 2006). In BFT, pathogens and diseases could be controlled more efficiently than the traditional methods such as using antibiotics, which led to an increase the resistance ability of pathogens (Defoirdt *et al*., 2004; Defoirdt *et al*., 2007). The effect of microbial communities on protecting aquatic species against the pathogenicity of *V. harveyi* has been reported to be interfering with the pathogen quorum sensing system (Crab *et al*., 2010). Increasing the immune system of cultured aquatic animals is another benefit of using BFT, which increases their resistance to diseases. One of the stimulants for the immune system is bacteria and their products. Therefore, the BFT which deals with bacteria and their compounds contains immunostimulants (Crab *et al*., 2012). Recently, BFT has experienced a growing trend in developed countries such as China, Japan, South Korea, the United States, etc. In Iran, it has not been used industrially so far. Considering the high potential and available resources in Iran, extensive research in this area and introducing it to the industry is necessary. Moreover, using this method in the interior brackish water and the saline and barren lands in the country makes shrimp and other species (e.g., tilapia and carp) farming possible in many provinces.

In the present study, it was aimed to investigate the effects of BFT on water quality indices, bacterial load of *Vibrio*, immune responses of shrimp, growth and survival rate in comparison with a traditional method as control group.

Materials and methods

Experimental design

This research was carried out on white-leg shrimps (*L. vannamei*) and performed at Iranian Fisheries Science Research Institute, Shrimp Research Center (Bushehr, Iran). The 5-gram shrimps provided by the Shrimp Research Center were used in this study. A 1000-liter fiberglass tank was used to produce biofloc and filled with filtered and sterilized sea water. A daily amount of shrimp manufactured food with 38% protein along with sugar as a carbohydrate source, were added to the tank to adjust the ratio of carbon to nitrogen. The carbon to nitrogen ratio was calculated using the Avnimelech method (Avnimelech, 1999). For inoculating of the bacteria to the tank, the soil of shrimp culture pond's floor was added to the tank. To examine the level of produced biofloc in water and measure the level of total suspended solids (TSS), an Imhoff funnel was used and then the shape of biofloc mass was observed using a light microscopy (Nikon, Japan) and recorded

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(Avnimelech and Kochba, 2009). To suspend the created particles and maintain the required oxygen in the system, intense aeration was performed using an aeration pump and several air stones in different parts of the tank. Three weeks was passed to produce the biofloc (Kim *et al*., 2014). Then, the water of this tank, as an original source, was used for the treatment tanks in the following stages of the experiment.

In this study, three biological replications were used for two experimental groups of BFT and a control system, with 100 individuals per each. In the control system, shrimps were cultured based on the traditional system and with routine water changes. In the control, about 30% of the water of each tank was drained once every two days and replaced with fresh, filtered and distilled seawater. Feeding was performed five times a day with manufactured food of Havoorash Company, based on the instruction of manufacturer. In the BFT, shrimps were cultured in the tanks containing biofloc without water exchange. Feeding was also performed five times a day. The water temperature was adjusted at 30-32°C and the photoperiod was maintained 14 h light and 10 h darkness.

Physicochemical factors of culturing water

Physicochemical factors of culturing water, temperature, salinity, pH, and dissolved oxygen were measured daily using a thermometer, salinity meter, pH meter (WTW, Winlab) and oxygen meter (WTW, Oxi 3210), respectively. To determine the levels of settled solids, one liter of the tank water was poured daily in an Imhoff cone and held for one h to settle the suspended particles. Then, the precipitated sediments were read and recorded in milliliters per liter (Avnimelech, 2012). To examine the biofloc density, the TSS was calculated in milligrams per liter. One hundred mL of the tank water was filtered by Whatman filter paper and dried in an oven at 105°C for 3 hours. Then, the papers weights before and after drying were used for calculating biofloc density (Azim and Little, 2008). Ammonia, nitrite and nitrate levels of water were measured once a week using a spectrophotometer (Unico 2150) and the values were calculated in mg/L with the standard diagram (ROPME, 1999; Madkour *et al*., 2020).

Growth and survival factors

Growth indices were calculated at the beginning and the end of the period with the following formulas (Tacon *et al*., 2002):

1. Weight gain= final weight (g)-initial weight (g)
2. Daily weight gain= final weight (g)-Initial weight (g) /culture days
3. Special growth rate: [(ln final weight (g) – ln initial weight (g)/time (day)] × 100
4. Food conversion ratio= weight gain (g) / feed given (g)
5. Survival rate= (numbers of final shrimps / number of initial shrimps) ×100 (Exposure to *V. harveyi* bacteria and registering survivors)

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sampling was performed before and after the challenge, at the beginning and the end of the rearing period, while the control and the BFT were exposed to *Vibrio harveyi.* After serial dilution, the inoculation was achieved on TCBS medium using spread plate method for 24 h at 30°C. The colony formed units (CFU) were counted and recorded on the counting screen. From one day before the beginning of the exposure day to the end of the rearing period, bacteria were daily counted to observe the growth and number of *Vibrio* bacteria.

Thirty days after beginning the experiment, the shrimp of both control and BFT were challenged with *V. harveyi* PTCC 1755 for 10 days. The pathogenic bacteria were isolated from contaminated shrimps identified using molecular methods (Mirbakhsh *et al*., 2014). Approximately 10^4-10^5 CFU/mL of *Vibrio harveyi* suspension was used for exposure in this study.

Immune system factors

After exposure to *V. harveyi*, some immune responses including the THC: total hemocyte; TPP (total plasma protein); PA (Phagocytosis activity) was determined., Shrimp hemolymph was collected using 1 mL syringe containing 0.4 Alsever anticoagulant solutions and transferred to a vial containing 0.4 anticoagulant solutions to determine THC (Kakoolaki *et al*., 2011). Accordingly, a 25 µL of the suspension was placed on a hemocytometer slide and the hemocytes were counted (Jiang and Zhou, 2004). The routine Biuret method was used to determine TPP (Acharya *et al*., 2004) and A 25 µL of the prepared hemolymph was transferred to a glass slide and incubated for three min at room temperature to measure phagocytic activity. The same value of bread yeast suspension containing 10^8 cells/ mL was added to the glass slide and kept at room temperature for 15 minutes. The slides were washed with the anticoagulant solution and fixed with 4% glutaraldehyde in anticoagulant solution for a min. Finally, the slides were washed with distilled water, fixed with ethanol, dried and stained with toluene blue for 5 min. The hemocytes were counted using a light microscope (Nikon, Japan) and digested yeast cells by the hemocytes were photographed and recorded (Jiang and Zhou, 2004; Liu and Chen, 2004).

Statistical analysis

The data were subjected to T-test (p-value<0.05) and analysis of variance (ANOVA), and the mean differences were compared using Duncan's range test at p-values≤0.05. All calculations were performed using IBM SPSS software, version 19. Regression test was used while dependent and independent variables were numerical.

Results

Physicochemical factors of water

The results showed significant differences (p<0.05) between the control and BFT for all the factors except for the pH, which decreased in the BFT (p<0.05) (Table 1). As

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expected, the length of culture period did not have any significant effect on the temperature, salinity and dissolved oxygen factors (p>0.05, data not shown) whereas a significant and gradually decreasing were clearly observed for the pH of the biofloc-treated water (p<0.05). The lowest pH values were recorded during the last days of the period (Fig. 1). It should be noted that all these values were in the optimal range (health and growth) for *L. vannamei* (Kuhn *et al*., 2010).

Table 1: Physicochemical factors of water in control and biofloc systems.

	Experimental Treatments		Statistical Differences
	Control	**Biofloc**	
Temperature	30.3 ± 1.0 -	30.2 ± 1.1 -	p>0.05
Salinity (g/L)	39.5 ± 0.5 (39.4 - 40.7)	39.8 ± 0.9 (39.9 – 40.8)	p>0.05
Dissolved Oxygen	8.3 ± 1.6 (10.2 – 6.7)	7.7 ± 1.8 (9.5 – 5.5)	p>0.05
pH	8.6 ± 0.1 [a] (8.7 – 8.4)	7.7 ± 0.2 [b] (8.2 – 7.3)	p<0.05

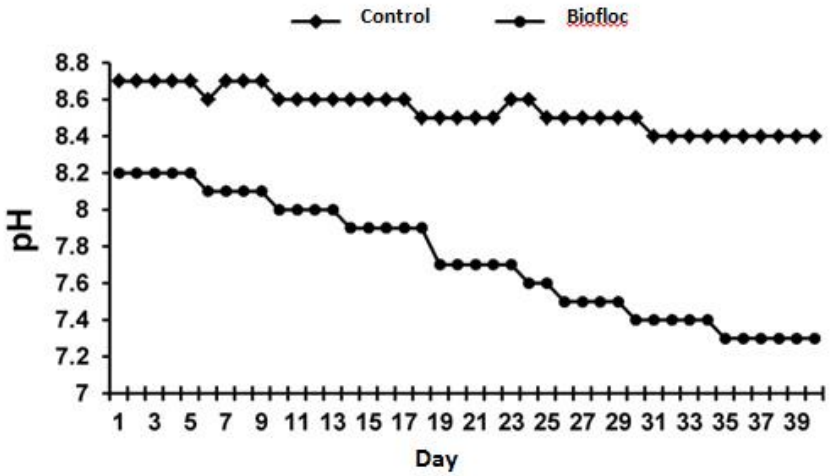


Figure 1: Periodic changes of pH in control and BFT during the husbandry period.

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Table 2: Levels of total ammonia, nitrite, nitrate, and total suspended solids in the control and BFT.

	Experimental Treatments		Statistical Differences
	Control	Biofloc	
Total ammonia	1.33±0.89 (2.41–0.09)	1.20±0.61 (2.21–0.09)	p>0.05
Nitrite	0.32±0.18 (0.54–0.01)	0.34±0.17 (0.57–0.04)	p>0.05
Nitrate	2.40±1.53[b] (4.27–0.24)	24.30±16.03[a] (40.97 – 0.43)	P<0.01
Total suspended solids	88.90±25.5[b] (130–39)	179.43±79.94[a] (315–59)	P<0.01

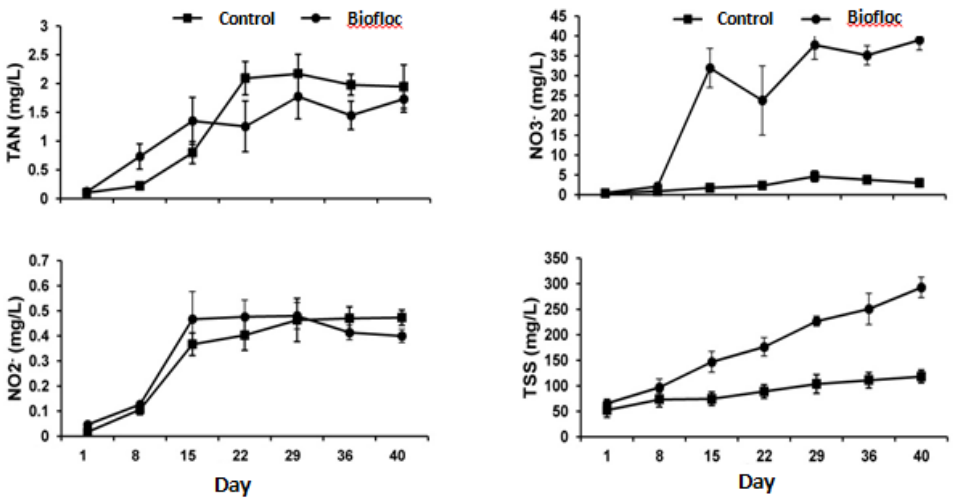


Figure 2: Periodic changes of total ammonia, nitrite, nitrate, and TSS levels in the control and BFT during the culturing period.

As the second phase, the weekly changes of nitrite, nitrate, total ammonia and TSS levels were considered. The results showed significant differences (p<0.05) between the control and BFT in nitrite and total ammonia contents. However, a significant increase in nitrate and TSS levels were recorded in BFT (p<0.001; Table 2) but due to the low toxicity of nitrate in comparison to ammonia and nitrite, it could be ignored for this valuable system. The interactions of two variables, the treatment and the time, were also significant for all of the mentioned factors (p<0.001) (Fig. 2).

Growth and survival factor

A significant increase in growth parameters was recorded for those cultured in BFT at the end of the rearing period. All growth indices including total body weight, total weight gain, daily weight gain, and SGR in BFT were significantly higher than those cultured in the control (p<0.05; Table 3). In addition, a significant lower ratio for feed conversion was obtained in BFT. It should be also noted that no significant differences of shrimp survival rates were recorded between both systems (p>0.05; Table 3).

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Load of Vibrio harveyi

At the beginning of the rearing period, no significant differences were observed in the density of *V. harveyi* in both control and BFT ($p>0.05$; control: 0.1×10^3 CFU/mL and BFT: 0.1×10^3 CFU mL^{-1}) while after 40 days, the density of *Vibrio* bacteria in BFT was significantly higher than the control ($p<0.01$; 2.5×10^3 and 1.2×10^3 CFU/mL, respectively). In addition, a significant positive correlation was recorded between *Vibrio* density and the levels of suspended solids in biofloc system ($p<0.01$; $R^2=0.95$).

Table 3: Growth and survival indices of white-leg shrimps cultured in control and biofloc systems (n =10)

	Experimental Treatments		Statistical Differences
	Control	Biofloc	
Final Weight	9.43 ± 0.42^b	11.13 ± 0.53^a	$p<0.01$
Weight Gain	6.91 ± 0.31^b	8.58 ± 0.23^a	$p<0.01$
Daily Weight Gain	0.172 ± 0.02^b	0.214 ± 0.03^a	$p<0.01$
Special Growth Rate	3.29 ± 0.08^b	3.68 ± 0.09^a	$p<0.01$
Food Conversion Ratio	1.72 ± 0.12^a	1.32 ± 0.11^b	$p<0.01$
Survival Rate	84.00±6.04	88.00±3.24	$p>0.05$

Challenge test

Following the induction of *V. harveyi*, its concentration increased ($p<0.05$) in both systems; which was higher in BFT (Fig. 3).

However, the survival rate of the shrimps cultured in both systems showed similar levels (Table 3).

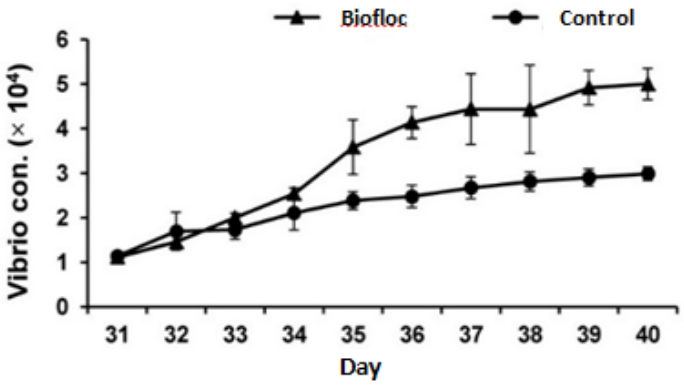


Figure 3: Periodic changes of *V. harveyi* bacteria colonies during 10 days (days 31-40 of the culturing period) of exposure in the control and BFT.

The amount of THC shrimps in BFT was significantly higher than those in the control ($p<0.05$). No significant difference was found in the TPP levels of shrimps ($p<0.05$), although an insignificant increase ($p<0.05$) was observed in the BFT (Fig. 4). In addition, shrimps in BFT showed higher values of hemocyte phagocytosis activity compared to the control group ($p<0.05$) (Fig. 5).

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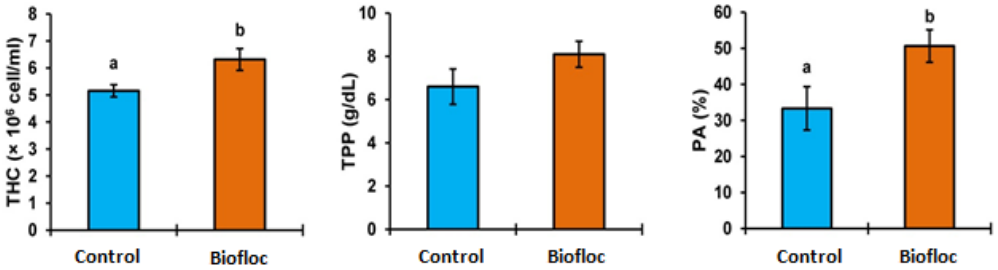


Figure 4: Immune system factors of white-leg shrimps after 10 days (days 31-40 of the culturing period) challenge with *V. harveyi* bacteria in the control and BFT (p<0.05, n=3). THC: total hemocyte; TPP: total plasma protein; PA: phagocytosis activity.

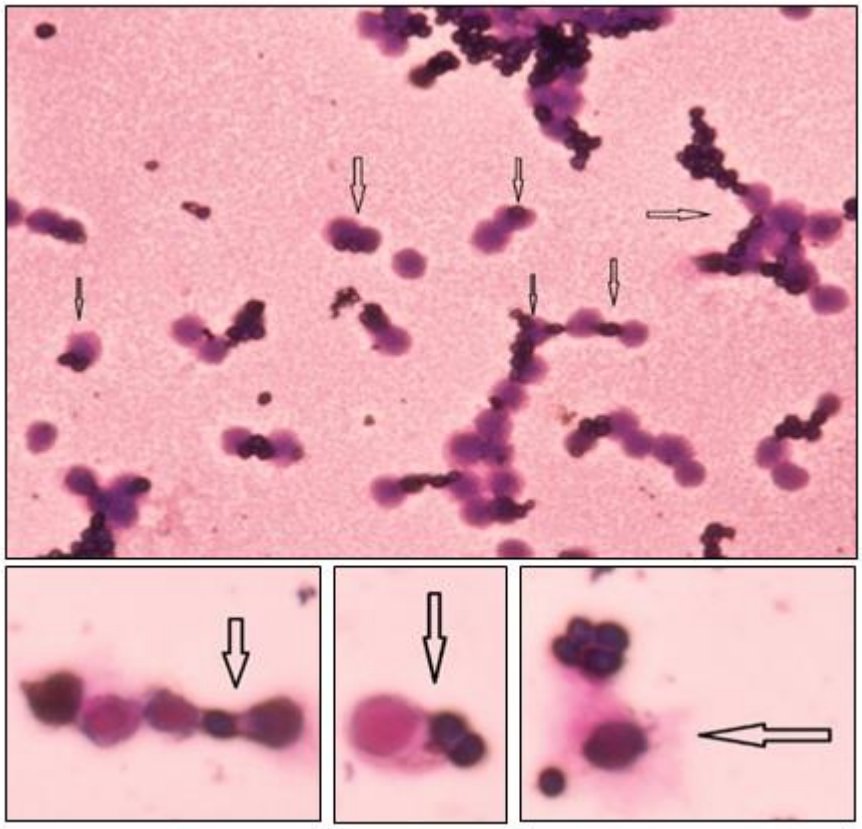


Figure 5: Phagocytosis activity of hemocytes in yeast cells ingesting.

Discussion

Investigating the biofloc-based culturing system as an efficient and modern technology in pacific white-leg shrimp (*L. vannamei*) culture in a zero-water exchange system is the purpose of this study. In the present study, the biofloc formation was properly performed by adding the appropriate amount of sugar as a carbon source. Based on the results obtained from the measured water-quality factors such as temperature, salinity and dissolved oxygen, there was a similar trend between control and BFT. Only the pH of the biofloc system showed significant decreases during the culturing period. Moreover, noteworthy results obtained from the study of levels of toxic nitrogen compounds in both

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systems, showed equality of total ammonia nitrogen and nitrite contents, representing high performance of BFT in removing toxic substances without water exchange. It should be noted that alteration in these Physicochemical factors of rearing water is dependent on the water sources entering the culturing tanks and the presence or absence of biofloc does not affect them. Previous research has also shown that the microbial activities related to BFT have no effect on the physical properties of water (Brito *et al*., 2014; Ahmad *et al*., 2019). Generally, the early stages of shrimp culturing were performed in the enclosed systems and the water physical factors were easily maintained in the optimum range for the optimal ranges f for Pacific white-leg shrimp juveniles.

High density of cultured shrimp in BFT leads to an increase in respiration rate and thus CO_2 concentration (Browdy *et al*., 2012). Increases in respiration rate and CO2 production are related to the conversion of ammonia nitrogen to microbial biomass by heterotrophic bacteria (Ebeling *et al*., 2006), which eventually lead to decreasing the pH of the system. It seems that the nitrification process in BFT could be one of the reasons of reduced pH in the current research. Interestingly, the much higher levels of nitrate content in BFT in comparison with the control may be clear evidence for it. The pH level affects all the chemical reactions of water and thus the physiological conditions of the shrimp (Zhang *et al*., 2017). Bacterial growth rate is also affected by pH changes (Ebeling *et al*., 2006). In culturing systems, the optimal pH range for white-leg shrimp is reported between 7 to 9 (van Wyk and Scarpa, 1999), which is also suitable for the heterotrophic and nitrifying bacteria growth, too (Ebeling *et al*., 2006).

Using sugar to increase the carbon to nitrogen ratio in the BFT resulted in maintaining toxic nitrogenous compounds at the low levels, which were suitable for shrimp culturing.
Generally, the nitrogenous compounds tend to increase. Total ammonia nitrogen can lead to mortality at concentrations more than 3.95 mg/L at salinity of 35 ppt (Lin and Chen, 2003). It has also been reported that nitrite concentrations more than 25.7 mg/L reduced shrimps' growth rate. According to the results of the current study, neither of these compounds reached critical concentrations and nitrite was even much lower. It seems that the adjusted nitrogen compound levels in the right range of shrimp growth and health may be a result of the high concentration of heterotrophic bacteria, algae, protozoa, silicates, rotifers, zooplanktons and organic materials attached to flocs. It should be noted that flocs are rich of nitrogen, phosphorus and carbon and thus, are a suitable environment for microbial growth (Zhao *et al*., 2014). It seems that no significant differences in ammonia and nitrite levels of both systems in this experiment are probably due to the large microbial biomass of the flocs, which has also been shown in previous

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studies (Avnimelech, 1999; Anand *et al*., 2014). Unfortunately, the type, species and density of the microorganisms attached to the flocs were not investigated in the present study. However, it is noteworthy that the previous studies on BFT have clearly shown that varieties of microorganisms were produced on the flocs (Kim *et al*., 2014). Therefore, it is expected that the flocs formed in this study are also full of variable microorganisms, which have had a positive effect on water quality.

Low nitrite concentrations in the BFT may indicate the oxidation of ammonia to nitrate (Cohen *et al*., 2005) and an efficient nitrification process in this system. An efficient nitrification process usually leads to increasing levels of nitrate (Ebeling *et al*., 2006), which is consistent with our results, which show the absorption by heterotrophic bacteria and oxidation to nitrate by nitrifying bacteria were the two major processes in this study.

Our results showed that the level of suspended solids (TSS) was significantly higher in the BFT compared to the control, especially from day 10. This is quite normal, because the heterotrophic bacteria production increases the TSS (Wang *et al*., 2015). Samocha *et al*. (2007) reported TSS concentrations of 275-800 mg/L (mean 379 mg/L) and a survival rate of 85.8% for white-leg shrimps cultured in zero-water exchange system (Samocha *et al*., 2007). They showed that short-term exposure of shrimp to concentrations of more than 500 mg/L of TSS had no destructive impacts. In a similar study, no destructive impacts were reported on post-larvae growth and survival rate under 613-762 mg/L concentration of TSS in biofloc system (Kim *et al*., 2014). It should be noted that the negative effect of TSS occurs through their sediments in the gills of cultured shrimp (Ray *et al*., 2011). The maximum amount of TSS in our study was 315 mg/L, which was less than the critical level (500 mg/L) for negative effects on the growth and survival rate of white-leg shrimps.

On growth and survival rates, our result showed that, the growth rate and nutritional parameters of white-leg shrimp in BFT were more than the control group. It seems that the cultured shrimps used the bioflocs as food. Other researchers have also shown that the BFT significantly increased the growth performance of the shrimp (Ballester *et al*., 2010). Reis *et al*. (2019) reported that young *L. vannamei* in the BFT showed a higher growth rate in comparison with the shrimps in a traditional system (Reis *et al*., 2019). Arnold *et al*. (2009) showed that adding a carbon source (cassava powder) for biofloc production significantly enhanced the growth rate of *Penaeus monodon* shrimp (Arnold *et al*., 2009). It is obvious that in the BFT different microbial communities were developed (Haslun *et al*., 2012) and thus be used as a food source for the shrimps (Kent *et al*., 2011). Many researchers have reported that increasing the levels of protein and fat in biofloc as a food source, reduced the feed conversion

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ratio (Megahed, 2011; Zhao *et al*., 2014). Avnimelech in 2006 showed that shrimp were fed the biofloc particles, which may lead to controlling the density of heterotrophic bacteria and other microorganisms and thus reducing waste and consumption of outer food (Avnimelech, 2006). As an important economical point, shrimps provide their protein needs by consuming bioflocs, so the protein content of the diet could be reduced (Browdy *et al*., 2012; Poli *et al*., 2019). More importantly, low-protein food is cost-effective and less harmful to the environment (Xu *et al*., 2016).

It has been reported that survival rates of the shrimps in BFT are more than traditional systems (Poli *et al*., 2019). Several researchers have reported a survival rate of about 85% (Xu and Pan, 2013; Furtado *et al*., 2015). However, in our results, the survival rate of control and BFT was similar. This is probably due to the experimental period, biosecurity and suitable and optimal conditions of the control system.

The density and concentrations of bacteria is directly related to the amount of organic material in water. In zero-water exchange systems like as BFT, the level of organic material is very high, which creates favorable conditions for the growth and development of bacteria communities such as Vibrio bacterial species (Ferreira *et al*., 2015). As expected, our results showed that the density of *Vibrio* bacteria (during 40 days of culturing period) in the biofloc system was significantly higher than the control system. Vibrio species predominately desire to grow in shrimp-dependent microbial communities (Balcázar *et al*., 2007) and cause harmful effects on shrimp's immune system. It is expected that in our experiment, the higher loads of vibrio bacteria in biofloc system in comparison with control system led to decreasing the shrimp survival rates while no significant differences were obtained between the two systems. This case is discussed below by details.

Exposing to the pathogens and the immune system

Based on our results, the load of *V. harveyi* bacterial was higher in the BFT compared to control system while the survival rate was similar. However, shrimp mortality in control treatment due to disease, was higher than biofloc system. More wounds on the body, especially in the tail were found in shrimps cultured in control system compared to biofloc system. According to the previous reports, *V. harveyi*, *V. parahaemolyticus*, *V. alginolyticus*, *V. anguillarum*, *V. vulnificus* and *V. splendidus* are the most endangered species of *Vibrio* which play important roles in shrimp disease and mortality (Brito *et al*., 2014). They form colonies in shrimp hepatopancreas and reduce shrimp growth. Interestingly the feed conversion ratio also increased for providing energy for enhanced physiological process (Vieira *et al*., 2013). It has been reported that biofloc systems can control the severity of

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pathogenicity of Vibrio species (Balcázar *et al*., 2007; Crab *et al*., 2010). The microorganisms and their product along with the nutrients that are available on flocs play key roles in improving the immune systems of the shrimps (Crab *et al*., 2010). In fact, natural probiotics formed in biofloc particles may struggle against pathogenic vibrio and other parasites, either internally or externally (Emerenciano *et al*., 2013). It is obvious that more survival rate in the biofloc system after exposure to more load of *V. harveyi* was due to the positive effects of bioflocs on shrimp immune system.

The shrimp health depends on environmental factors and stressful environment reduces the immunity of shrimps (Matozzo *et al*., 2011). It has been reported that the health status and immune system function of shrimp in the BFT has increased due to improved water quality and biofloc particles (Xu and Pan, 2013; Ekasari *et al*., 2014).

Phagocytosis has also been considered as the main and important process in inhibiting the microorganisms (Bachère, 2000). Also, the amount of plasma protein in shrimp is a proper indicator for nutritional status, which is directly related to immune system function and health (Song *et al*., 2003). In our study, a significant increase in total hemocyte count (THC) and hemolymph phagocytosis activity (PA) was observed in BFT. It has been reported that all these three factors decreased in shrimps' blood when culturing system infected by pathogens (Song *et al*., 2003). Therefore, the higher levels of THC and PA in the hemolymph of BFT shrimps indicate the higher level of health and strength against pathogens. Xu and Pan (2013) showed that the number of hemocytes increased in BFT and reported that biofloc-cultured shrimps had higher levels of antioxidant activity in their plasma and hepatopancreas (Xu and Pan, 2013). It should be noted that biofloc is not only a source of nutrients such as proteins, fats, minerals and vitamins (Izquierdo *et al*., 2006; Xu *et al*., 2016), it also provides an abundance of natural microbes and bioactive compounds such as fat-soluble pigments and vitamins and other immune-stimulating compounds (Crab *et al*., 2012) that improve the immune system function of cultured shrimps. The important mechanisms of BFT involved in improving the shrimp performance have been reviewed in some studies (Farzanfar, 2006; Ninawe and Selvin, 2009; Ekasari *et al*., 2014; Poli *et al*., 2019). They point to the mechanisms of immunomodulation, competitive inhibition, biological repair, nutrient supply, and enzymatic activity in digestion and inhibition of quorum sensing of bioflocs. However, further studies are needed to determine the exact mechanisms involved in the role of biofloc in the shrimp immune system.

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