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Research Article

Evaluation of High Stocking Densities on the Water Quality and Growth Performance of Pacific White Shrimp *Litopenaeus vannamei* Reared in a Mixotrophic Biofloc Nursery System

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The present study aimed to explore the effects of intensive culture densities on the water quality and growth performance of the Pacific white shrimp *Litopenaeus vannamei* reared in a mixotrophic biofloc system (heterotrophic/chemoautotrophic) at three stocking rates of 5,000, 4,000, and 3,000 PL/m^3 (namely G5000, G4000, and G3000, respectively) for 21 days. At the end of the study, the mean values of water temperature, dissolved oxygen, dissolved oxygen saturation level, pH, oxidation-reduction potential, free carbon dioxide, alkalinity, total ammonia nitrogen (TAN), nitrite-nitrogen (NO_2-N), nitrate-nitrogen, and settleable solids did not substantially differ among study groups ($p > 0.05$). In addition, the highest mean levels of TAN and NO_2-N recorded were 1.27 mg/l (G4000) and 9.23 mg/l (G3000), respectively. The results of growth performance revealed that final body weight, weight gain, weekly growth rate, and specific growth rate were not affected by the studied densities ($p > 0.05$). Although no significant differences were observed ($p > 0.05$), the G3000 treatment exhibited the highest survival rate (80.66% ± 8.56%), followed by G4000 (75.05% ± 0.31%) and G5000 (71.63% ± 2.11%). However, the shrimp yield was meaningfully higher for G5000 treatment (1.72 ± 0.0.09 kg/m^3, 0.69 ± 0.03 kg/m^2) compared with G3000 group (1.27 ± 0.0.12 kg/m^3, 0.48 ± 0.04 kg/m^2) ($p < 0.05$). Our results indicate *L. vannamei* can be reared at intensive nursery densities of up to 5,000 PL/m^3 (2,000 PL/m^2) until reaching 0.5 g without significant retardation in water quality, growth, and survival.

1. Introduction

The aquaculture sector furnishes the human population with an alternative avenue to address food security and meet protein demand [1]. There is an increased demand for aquaculture production to sustain the requirements of humanity, which mandates more appropriate strategies such as intensification to increase output without a significant increase in natural resources (land and water) exploitation [2]. Biofloc technology (BFT) is a potential alternative technique that allows the intensive culture of aquatic animals along with reduced water use [3, 4].

The nitrogen compounds in the BFT systems with limited discharge are controlled by the combination of heterotrophic and chemoautotrophic activity [2, 5, 6]. The heterotrophic and chemoautotrophic dominance depends on the carbon-to-nitrogen (C/N) ratio in the culture unit. A high C/N ratio through daily addition of organic C sources boosts heterotrophic growth transforming nitrogenous wastes (75%–90% of feed N) into single-cell protein hence maintaining a good water quality [2, 5]. However, chemoautotrophs are predominant in the mixotrophic BFT systems (heterotrophic/chemoautotrophic), where organic carbon fertilization occurs to prevent total ammonia nitrogen (TAN) from reaching higher levels (>0.5–1 mg/l) [7, 8]. Hence, chemoautotrophs are responsible for long-term nitrogen control in mixotrophic systems. Nowadays, mixotrophic BFT systems are adopted since they enhance shrimp growth and survival, reduce solids

generation and water consumption, and need less organic C supplementation, thereby reducing production costs [9, 10].

The nursery phase is an intermediate step between the hatchery and the grow-out phase [11]. It has the advantages of improving growth performance, survival, and feeding efficiencies during the latter stage [8]. In the nursery stage shrimps are often subjected to intensive densities to optimize space and energy [11] which generally increases stress and mortalities, due to low water quality, unbalanced artificial feed, disease episodes, and cannibalism [3, 7, 12–14]. Recent studies documented the possibility of intensifying the Pacific white shrimp *Litopenaeus vannamei* farming in the BFT system [7, 12, 15–17]. BFT enables intensification by providing a high-quality supplemental food source for shrimp supplying protein and other necessary nutrients required for growth and survival [3, 6, 18, 19]. BFT also improves immune responses and disease resistance via its natural probiotic/prebiotic effects [20–25].

Based on the above considerations, the present study aimed to evaluate the potential effect of different intensive stocking densities on the water quality and productive performance of *L. vannamei* during the nursery stage in a mixotrophic BFT system.

2. Materials and Methods

2.1. Water Preparation. This study was performed in nine circular fiberglass tanks (working volume 200 l, bottom surface area 0.5 m^2) filled with pretreated (chlorine solution 10 ppm, neutralized using sodium thiosulfate 25 ppm) seawater water (salinity 34 ppt, temperate 30°C, pH 7.9, dissolved oxygen (DO) 5.7 mg/l). To promote biofloc formation, all the experimental tanks were supplied with 5.5 g milled shrimp commercial feed (Daneh Talaei Kamijan Co., Iran), 4.69 g rice bran, 3 g commercial probiotic (*Bacillus subtilis* IS02, 2.5×10^{12} CFU/kg; Takcell®, Takgene Zist Co., Iran), 3 g yeast (*Saccharomyces cerevisiae*, 2×10^9 CFU/g; Keshtsabz Co., Iran), 2.26 g sodium bicarbonate ($NaHCO_3$; Neutron pharmachemical Co., Iran), 9.36 g white sugar, and 4 l of water containing bioflocs from grow-out tanks (TAN < 0.00 mg/l, NO_2-N < 0.25 mg/l, NO_3-N < 1.1 mg/l, Alkalinity 131–150 mg/l $CaCO_3$, settleable solids (SS) <6.5 ml/l) 2 days before shrimp stocking. The calculated input for the C/N ratio was 23.75 : 1, as per the calculations provided by Avnimelech [2]:

$$\begin{aligned}&\text{Carbon from sugar: } 9.36\text{ g} \times 0.4\ (40\%\text{ organic carbon}) = 3.74\text{ g; Carbon from feed: } 5.5\text{ g} \times 0.5\ (50\%\text{ organic carbon})\\&= 2.75\text{ g; Nitrogen from feed: } 5.5\text{ g} \times 0.4\ (40\%\text{ protein}) \times 0.16\ (16\%\text{ nitrogen in protein})\\&= 0.352\text{ g; Carbon from rice bran: } 4.69\text{ g} \times 0.4\ (40\%\text{ organic carbon}) = 1.87\text{ g; Total carbon: } 3.74 + 2.75 + 1.87\\&= 8.36\text{ g; C/N ratio: } 8.36/0.352 = 23.75.\end{aligned} \quad (1)$$

While bacteria may have difficulty accessing carbon from complex sources like rice bran within a short preparation time of 2 days, incorporating rice bran provides crucial vitamins and minerals necessary for bacterial growth and flocculation. Without considering the carbon contribution from rice bran, the initial C/N ratio would be 15.9.

2.2. Shrimp and Experimental Design. Pacific shrimps (*L. vannamei*) (PL7–9; 1.06 ± 0.32 mg; 7.23 ± 0.63 mm) were purchased from a commercial hatchery in Qeshm, Hormozgan, Iran. The laboratory received the shrimp in plastic bags that were placed inside a Styrofoam box. The study was performed in the indoor aquaculture facilities of the Persian Gulf and Oman Sea Ecological Research Center (Bandar Abbas, Iran). Shrimp acclimation to the lab conditions was conducted in 300 l fiberglass tanks for 5 days. The daily feeding rate was 70% of the estimated biomass (40% crude protein; Daneh Talaei Kamijan Co®., Iran) [7]. Fifty percent of tank volume was exchanged daily to keep the levels of DO (5.8–6.2 mg/l), salinity (32–35 ppt), temperature (29–31°C), and TAN (<0.2 mg/l) during this period. Shrimp weighing 3.18 ± 2.08 mg and measuring 11 ± 1.58 mm in length were transferred into experimental tanks and stocked at the rates of 5,000 PL/m^3 (G5000), 4,000 PL/m^3 (G4000), and 3,000 PL/m^3 (G3000), resulting in three treatments in triplicate. The study lasted for 21 days.

Shrimp were fed a commercial diet with 40% crude protein (Daneh Talaei Kamijan® Co., Iran) three times daily at 7:00, 13:00, and 22:00. The initial daily feeding rate was 30% of body weight, which gradually reduced to 17% at the end of the trial [26]. The alkalinity and pH levels were maintained using a mixture of $NaHCO_3$ and calcium hydroxide ($CaOH_2$) (9 : 1 ratio) at the rate of 10%–15% of the daily feed input [7, 27]. The water temperature was natural at approximately 31 ± 1°C. Two 25 mm round air stones were connected to the air compressor (75 W; Haila® Co., China) and placed at the center of each tank for air supply (DO > 6 mg/l) and creating sufficient turbulence to keep bioflocs in suspension. Once a week, 2 g of a commercial probiotic (Takcell®, Takgene Zist Co., Iran) were used in each tank. No water was renewed during the experiment, though dechlorinated tap water was added to compensate for the evaporation. The concentrations of solids were controlled by filtering water from tanks (days 13 and 19; 20–30 l of water) through a fine mesh net (~200 microns) into a bucket and returning the filtrate water to the corresponding tank. Floc volume may not provide a comprehensive assessment of the actual solids present in the system [2], so removal of solids occurred regardless of the floc volume in order to prevent the accumulation of harmful organic matter at the bottom of the tank and the formation of anaerobic zones. When the TAN concentrations surpassed 0.5 mg/l, the white sugar as the organic carbon source was mixotrophic

with the water in a beaker and distributed evenly in each tank (at 10 a.m.). The amount of sugar was calculated as per Samocha [7] and Brandão et al. [9]:

$$\text{Sugar (g)} = \frac{[\text{TAN (mg/l)} \times \text{Water volume (l)} \times 0.001\ (\text{converting mg to g}) \times 6\ (\text{desired C/N ratio})]}{0.40\ (\%\ \text{Carbon in sugar, decimal value})}. \quad (2)$$

2.3. Physico-Chemical Parameters of Water. Water quality parameters such as temperature, DO, pH, salinity, pHmV, DO saturation, oxidation-reduction potential (ORP), electrical conductivity (EC), total dissolved solids (TDS), and seawater specific gravity (SSG) were monitored daily (11 a.m.) using a portable water quality analyzer (model AP2000; Aquaread Ltd®., UK). The levels of total ammonia nitrogen, nitrite-nitrogen (NO_2-N), and alkalinity were estimated as per the standard procedures [28]. Nitrate-nitrogen (NO_3-N) levels were determined following Strickland and Parsons [29]. The estimation of free carbon dioxide (CO_2) concentration was made following Reis et al. [30]. The concentration of SS was measured using Imhoff cones [7].

2.4. Shrimp Performance. At the end of the experiment, shrimp from all tanks were harvested and then weighted using an electric balance (BP12002; Biobase Co., China) accurate to two decimal places. The mean final body weight (FBW, g), weight gain percentage (WG %), weekly growth rate (WGR), specific growth rate (SGR %/day), feed conversion ratio (FCR), survival rate (SR %), and yield were determined using the formulas:

$$\text{FBW (g)} = \frac{\text{Biomass of shrimp harvested (g)}}{\text{Number of shrimp}}, \quad (3)$$

$$\text{WG (\%)} = \frac{(\text{Final weight (g)} - \text{Initial weight (g)})}{(\text{Initial weight (g)})} \times 100, \quad (4)$$

$$\text{WGR (g/week)} = \frac{(\text{Final weight (g)} - \text{Initial weight (g)})}{(\text{Days of the experiment}/7)}, \quad (5)$$

$$\text{SGR (\%/day)} = \left[\frac{(\ln \text{final weight (g)} - \ln \text{initial weight (g)})}{\text{Days of the experiment}}\right] \times 100, \quad (6)$$

$$\text{FCR} = \frac{\text{Feed provided (g)}}{(\text{Final weight (g)} - \text{Initial weight (g)})}, \quad (7)$$

$$\text{SR (\%)} = \left(\frac{\text{Total no. of shrimp harvested at the end}}{\text{No. of shrimp stocked)}}\right) \times 100, \quad (8)$$

$$\text{Yield (kg/m}^3\text{)} = \frac{\text{Harvested biomass (g)}}{\text{Culture water volume}}. \quad (9)$$

2.5. Statistical Analysis. The results of Shapiro–Wilk and Levene's tests were checked for normality and homoscedasticity of the data. Then, repeated measures one-way analysis of variance (ANOVA) followed by Tukey's test was carried out to explore the significant differences among the treatment at $p<0.05$. Statistical analysis of the present study data (mean ± standard error) was conducted using SPSS v22 for Windows (IBM Co., USA).

3. Results

3.1. Water Quality. The mean values of water quality parameters monitored in different groups during 21 days of *L. vannamei* intensive nursery culture are presented in Table 1. Based on the results showed that water variables including temperature, DO, dissolved oxygen saturation level, pH, ORP, and free CO_2 did not substantially differ among different groups ($p>0.05$). However, the levels of salinity, TDS, and EC were found to be significantly higher for G3000 treatment compared with the other groups ($p<0.05$). No significant differences were revealed between study groups concerning alkalinity, TAN, NO_2-N, NO_3-N, and SS mean levels ($p>0.05$).

According to Figure 1, TAN concentrations accumulated gradually from the beginning of the study. TAN levels were <0.4 mg/l during the three initial days of the experiment. The highest mean levels of TAN for G5000 and G3000 occurred on the 4th day (1.08 and 0.57 mg/l, respectively). However, G4000 treatment recorded TAN peak mean levels of 1.16 and 1.27 mg/l on the 6th and 7th days, respectively. TAN levels then declined rapidly to <0.00 mg/l on the 8th day and were generally <0.1 mg/l until the end of the study. The repeated measures ANOVA revealed that TAN levels on the 4th day were significantly higher than those of 2nd, 8th, 9th, 11th, 12th, and 16th–21st days. In addition, significant differences were observed between the 3rd and 19th days, as well as between the 5th and 13th, 15th and 21st days of the experiment.

The levels of NO_2-N fluctuated throughout the experiment (Figure 2). The NO_2 concentrations elevated from 0.14 mg/l on the 3rd day to 5.82 mg/l (G5000 and G4000 groups) and 9.23 mg/l (G3000 group) on the 5th day of the study. There was a substantial decline in NO_2-N levels during the 13th day (<0.19 mg/l) due to solids removal.

Table 1: The results (mean ± SE, minimum, and maximum) of water quality monitoring in different treatments after 21 days of *L. vannamei* intensive culture during the nursery phase.

Parameters	Treatments			*p*-Value
	G5000	G4000	G3000	
Temperature (°C)	31.66 ± 0.17 (29.4, 32.9)	32.00 ± 0.14 (29.6, 32.9)	32.10 ± 0.13 (29.7, 32.9)	0.118
Salinity (ppt)	34.78 ± 23[b] (31.68, 36.55)	34.57 ± 16[b] (33.06, 36.55)	35.71 ± 23[a] (33.36, 37.89)	0.001
DO (mg/l)	6.07 ± 0.08 (4.83, 6.61)	6.04 ± 0.05 (5.37, 6.63)	6.05 ± 0.04 (5.60, 6.57)	0.953
DOS (%)	97.71 ± 1.18 (79.20, 104.10)	98.10 ± 0.74 (88.50, 103.80)	98.92 ± 0.56 (92.10, 103.70)	0.609
Free CO_2 (mg/l)	4.27 ± 0.64 (2.99, 7.17)	3.86 ± 0.43 (2.60, 5.33)	3.84 ± 0.41 (2.42, 5.32)	0.801
pH	7.78 ± 0.02 (7.58, 8.01)	7.81 ± 0.02 (7.62, 8.05)	7.84 ± 0.01 (7.66, 8.06)	0.172
pHmV	−67.65 ± 1.64 (−81.7, −55.60)	−69.51 ± 1.48 (−83.7, −57.80)	−71.80 ± 1.12 (−84.3, −60.4)	0.128
TDS (mg/l)	31,205 ± 188[b] (28,691, 32,608)	31,034 ± 135[b] (29,840, 32,604)	31,941 ± 187[a] (30,076, 33,690)	0.001
EC (μS/cm)	48,008 ± 290[b] (44,140, 50,167)	47,745 ± 208[b] (45,908, 50,160)	49,141 ± 288[a] (46,272, 51,832)	0.001
ORP (mV)	86.54 ± 3.75 (65, 149.80)	84.31 ± 3.36 (61.70, 142)	82.91 ± 3.17 (59.50, 136.20)	0.754
SSG (sigma-t, σ_T)	21.88 ± 0.27[ab] (20.2, 23.50)	21.67 ± 0.17[b] (20.41, 23.30)	22.44 ± 0.26[a] (20.66, 24.30)	0.076
Alkalinity (mg/l $CaCO_3$)	128.31 ± 7.87 (70.59, 158.62)	131.79 ± 5.34 (86.27, 144.83)	135.13 ± 4.36 (109.8, 158.62)	0.729
TAN (mg/l)	0.21 ± 0.05 (0.00, 1.5)	0.22 ± 0.0.05 (0.00, 1.34)	0.11 ± 0.0.02 (0.00, 0.65)	0.225
NO_2-N (mg/l)	4.12 ± 0.96 (0.06, 9.15)	4.56 ± 1.08 (0.02, 11.03)	3.97 ± 0.98 (0.06, 9.23)	0.913
NO_3-N (mg/l)	9.74 ± 2.15 (0.09, 15.96)	9.02 ± 2.29 (0.14, 15.17)	8.10 ± 2.41 (0.05, 14.42)	0.881
SS (ml/l)	3.34 ± 1.21 (0.1, 8.50)	2.68 ± 0.94 (0.1, 6.50)	1.18 ± 0.96 (0.1, 2.80)	0.062

Note: Treatments included: G5000, 5,000 PL/m^3; G4000, 4,000 PL/m^3; G3000, 3,000 PL/m^3. Different superscript letters imply significant differences at $p < 0.05$. DO, dissolved oxygen; DOS, dissolved oxygen saturation level; TDS, total dissolved solids; EC, electrical conductivity; ORP, oxidation-reduction potential; SSG, seawater specific gravity; TAN, total ammonia nitrogen; SS, settleable solids.

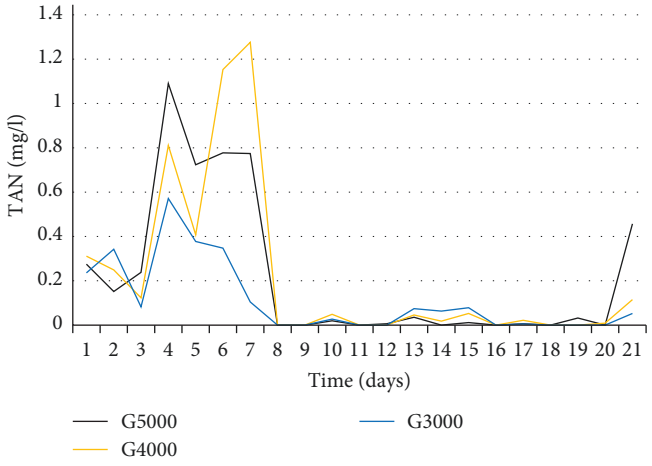

Figure 1: Variations in total ammonia nitrogen (TA-N) concentrations during 21-day *L. vannamei* intensive culture. G5000, 5,000 PL/m^3; G4000, 4,000 PL/m^3; G3000, 3,000 PL/m^3.

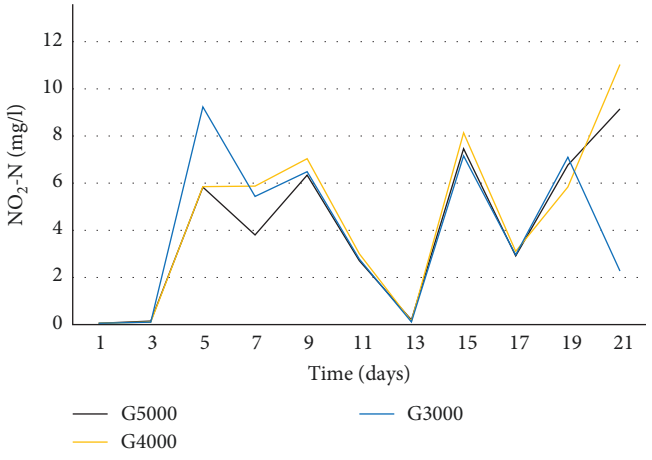

Figure 2: Changes in NO_2-N concentrations during 21-day *L. vannamei* intensive culture. G5000, 5,000 PL/m^3; G4000, 4,000 PL/m^3; G3000, 3,000 PL/m^3.

However, NO_2-N concentration again raised significantly on the 15th day.

The changes in NO_3-N concentrations during the study are given in Figure 3. Accordingly, the peak levels of NO_3-N occurred on the 5th day of the experiment (G5000, 15.96 mg/l; G4000, 15.16 mg/l; G3000, 14.42 mg/l). It then gradually declined until the 13th day, when solids removal led to decreased NO_3-N levels to 9.86, 6.63, and 4.76 mg/l in G5000, G4000, and G3000 treatments, respectively. NO_3-N fell to <6 mg/l in G4000 and G3000 treatments on the 21st day. However, NO_3-N levels in G5000 group were relatively constant (~10 mg/l) during the 13th and 21st days of the experiment.

The alkalinity levels were generally constant (136.2–143.1 mg/l $CaCO_3$) during the initial 9 days (Figure 4). The supplementation of $NaHCO_3$ and $CaOH_2$ was halted for a duration of 3 days (days 16–18) to determine if there is a well-established chemoautotrophic community that requires alkaline agents. Consequently, alkalinity levels decreased by day 19 of the study. Shortly after $NaHCO_3$ and $CaOH_2$ additions were resumed, alkalinity restored. According to the repeated measures ANOVA, the alkalinity levels on day 19 showed a significant difference when compared to the levels on days 1, 3, 5, and 9.

The SS concentrations elevated over time in all experimental groups (Figure 5). The SS concentration slowly

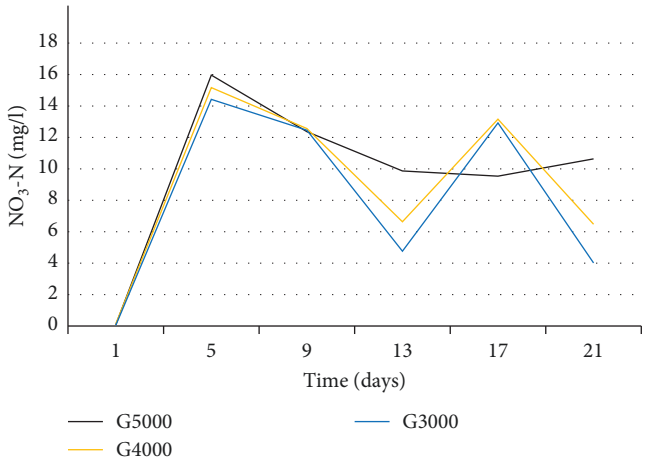

FIGURE 3: Variations in NO_3-N concentrations during 21-day *L. vannamei* intensive culture. G5000, 5,000 PL/m^3; G4000, 4,000 PL/m^3; G3000, 3,000 PL/m^3.

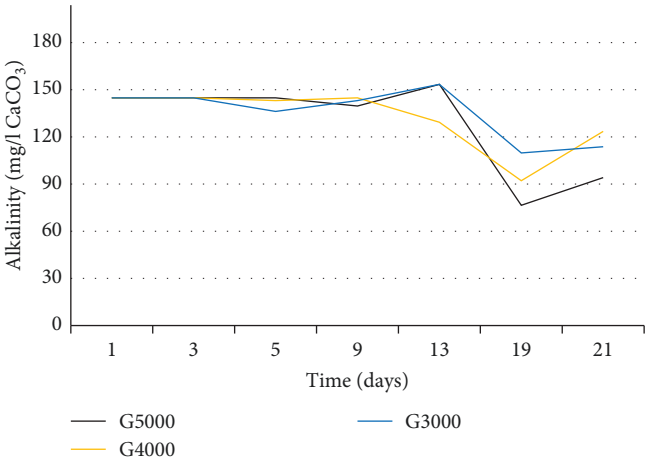

FIGURE 4: Alkalinity levels fluctuations during 21-day *L. vannamei* intensive culture. G5000, 5,000 PL/m^3; G4000, 4,000 PL/m^3; G3000, 3,000 PL/m^3.

increased during the initial 13 days of the experiment (<1.5 ml/l). The differences between all treatments from days 1 to 13 were not significant. The SS, however, rapidly elevated to 2.2 ml/l (G3000 group) and 6.5 ml/l (G5000 and G4000 groups) on the 16th day. This continued until the 19th day for G5000 and G3000 groups. The solids removal during the 13th day had no marked effect on decreasing SS levels. During the study, the highest and the lowest levels of SS were recorded for G5000 (8.5 ml/l) and G3000 (2.8 ml/l) treatments, respectively.

3.2. Growth. The results of growth performances and yield of *L. vannamei* under different intensive densities during the 21-day nursery phase are presented in Table 2 and Figure 6. According to the results, the FBW, WG %, WGR, and SGR %/day were not affected by the studied densities ($p > 0.05$). The lowest FCR (0.78 ± 0.08) was found for shrimp reared in G3000 treatment. However, there were no differences between study groups concerning FCR ($p > 0.05$). A higher survival rate

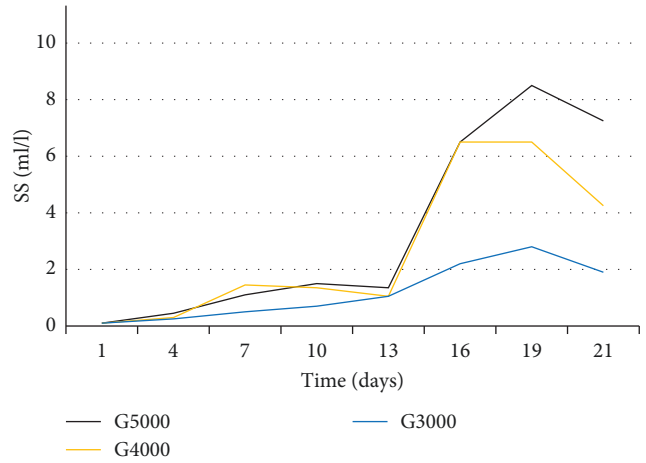

FIGURE 5: Changes in settleable solids (SS) concentration during 21-day *L. vannamei* intensive culture. G5000, 5,000 PL/m^3; G4000, 4,000 PL/m^3; G3000, 3,000 PL/m^3.

($80.66\% \pm 8.56\%$) was recorded in G3000 treatment and it was followed by G4000 ($75.05\% \pm 0.31\%$) and G5000 ($71.63\% \pm 2.11\%$), respectively. However, no marked differences were shown for experimental groups in terms of survival rate ($p > 0.05$). The shrimp yield was meaningfully higher for G5000 treatment (1.72 ± 0.09 kg/m^3) compared with G3000 group (1.27 ± 0.12 kg/m^3) ($p < 0.05$). Meanwhile, shrimp yield in G4000 treatment (1.41 ± 0.06 kg/m^3) was statistically similar to the other groups ($p > 0.05$).

4. Discussion

The levels of main water quality parameters monitored during 21 days of the present study were within the reported recommended range (temperature 26–37°C, pH 7–9, DO >5 mg/l, salinity 33–40 ppt, free $CO_2 < 20$ mg/l, and ORP 62–162 mV) for the growth and survival of *L. vannamei* [7, 26, 31, 32]. No significant differences were found between the groups examined.

Salinity in seawater measures the total amount of dissolved salts, while EC is related to the concentration of dissolved ions and TDS is a measurement of the total amount of organic and inorganic substances. SSG measures its density compared to pure water. As salinity increases, so do EC and TDS because they are affected by the presence of dissolved salts. In addition, when TDS increases, SSG also increases due to the increased density from the dissolved solids [33, 34]. The key disparity observed among the various treatments in the present study pertains to the different rates at which solids were removed and freshwater was added to maintain optimal salinity levels.

The alkalinity levels were not affected by the stocking density. The results demonstrated that mean levels of alkalinity in all groups were consistently 125–135 mg/l $CaCO_3$, which falls within the optimum range for *L. vannamei* and the bacterial community [5, 27]. Alkalinity indicates the concentration of titratable bases, mainly bicarbonate (HCO_3) and carbonate (CO_3^{-2}), in the culture water which can react

Table 2: The results (mean ± SE.) of *L. vannamei* (3 mg initial weight) growth performance and yield after 21 days of intensive culture during nursery phase.

Parameters	Treatments			*p*-Value
	G5000	G4000	G3000	
FBW (g)	0.48 ± 0.01	0.46 ± 0.02	0.49 ± 0.002	0.523
WG (%)	15966 ± 386	15558 ± 787	16471 ± 72	0.523
FCR	0.86 ± 0.04	0.86 ± 0.04	0.78 ± 0.08	0.566
WGR (g/week)	0.15 ± 0.005	0.15 ± 0.01	0.16 ± 0.001	0.523
SGR (%/day)	24.18 ± 0.11	24.05 ± 0.23	24.33 ± 0.02	0.525
SR (%)	71.63 ± 2.11	75.05 ± 0.31	80.66 ± 8.56	0.527
Yield (kg/m^3)	1.72 ± 0.09^a	1.41 ± 0.06^{ab}	1.27 ± 0.12^b	0.092

Note: Treatments included: G5000, 5,000 PL/m^3; G4000, 4,000 PL/m^3; G3000, 3,000 PL/m^3. Different superscript letters imply significant differences at $p < 0.05$. FBW, final body weight; FCR, feed conversion ratio; WGR, weekly growth rate; SGR, specific growth rate; SR, survival rate.

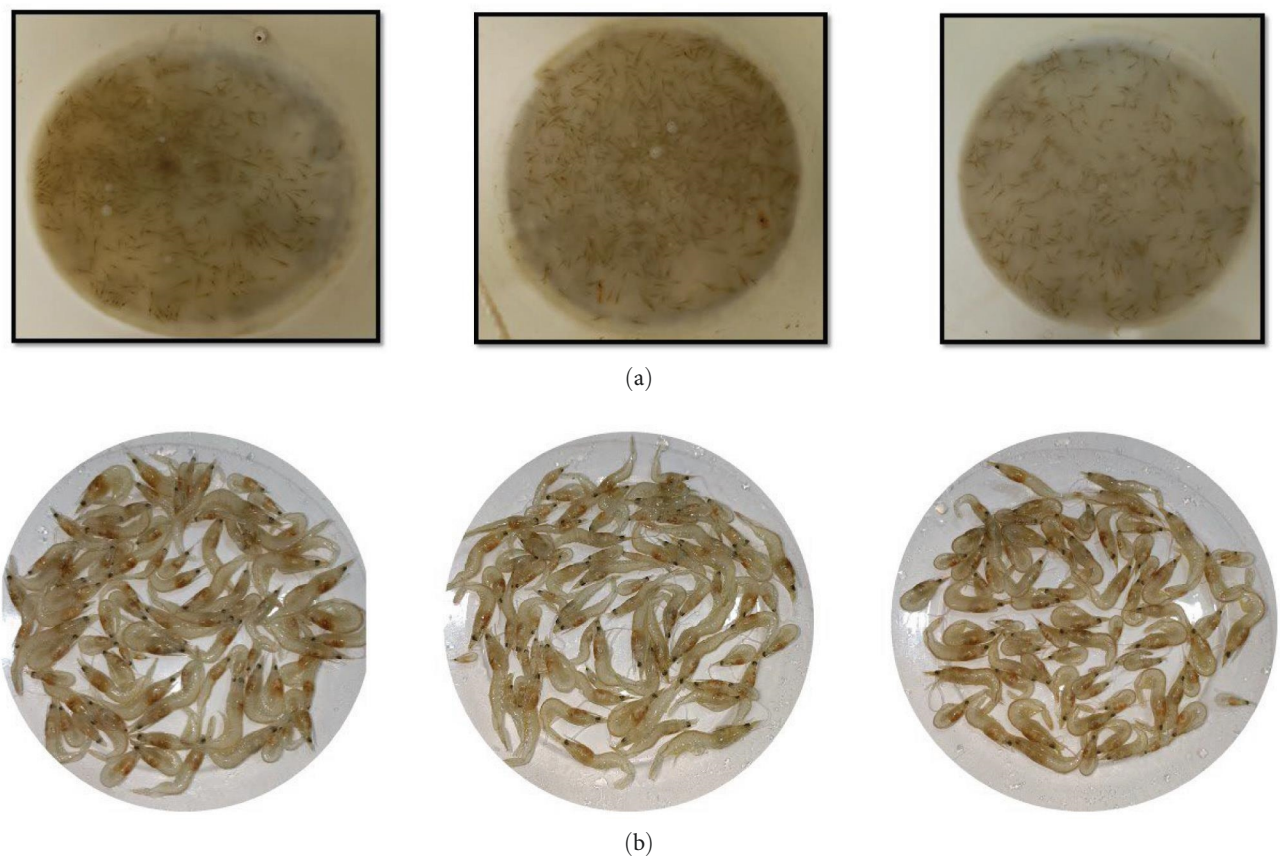

Figure 6: Top view of the study tanks (a) and shrimp (b) at the end of the study. From left to right: G5000, 5,000 PL/m^3; G4000, 4,000 PL/m^3; G3000, 3,000 PL/m^3.

with and neutralize hydrogen cations. Alkalinity is considered an important water quality parameter to maintain a steady pH level in the culture unit. Several other studies documented the reduction in alkalinity levels in the BFT systems [6, 9, 35, 36]. Different chemicals such as $NaHCO_3$ and $CaCO_3$ are commonly used to maintain alkalinity levels in BFT systems [27]. For example, Vinatea et al. [36] used $NaHCO_3$ when alkalinity <60 mg/l $CaCO_3$. Reports suggest that $CaCO_3$ alone did not prove effective in increasing alkalinity levels. However, a combination of $CaCO_3$ and $NaHCO_3$ was discovered to effectively elevate both alkalinity and pH [37]. In agreement, Samocha and Prangnell [38] suggested that the combined use of $NaHCO_3$ and $CaCO_3$ was efficient in increasing the alkalinity and pH in the BFT system, consistent with the results of the current study. The alkalinity levels experienced a significant decline in the current study when the use of alkaline agents was discontinued between the 16th and 18th day of the experiment. However, by reintroducing these specific chemicals on day 19, alkalinity levels were successfully regulated. The alkalinity reduction in mixotrophic BFT systems (heterotrophic/chemoautotrophic) is ascribed to the consumption of inorganic carbon (HCO_3)

by the bacterial community [5]. The stoichiometric analysis of bacterial metabolism showed that 7.14 and 3.5 g of alkalinity as $CaCO_3$ is depleted by chemotrophic and heterotrophic bacteria, respectively, per gram of NH_4-N [5].

The ammonia-oxidizing bacteria (AOB) population in the experimental tanks took a week to reach an adequate level. As a result, the culture units experienced moderately high TAN levels during the initial days of study. The elevated values observed between the 3rd and 8th day could possibly be attributed to fertilization of tank during preparation (352 mg N/200 l = 1.74 mg/l N), considering that the entire mixture was added a mere 2 days before the introduction of shrimp into the tanks. This accords with the results of Ekasari et al. [39]. However, organic carbon supplementation during this period effectively controlled TAN levels (<1 mg/l). Organic carbon fertilization in mixotrophic systems is specified to the first days of culture in order to induce TAN removal (when >0.5 mg/l) by heterotrophic bacteria until maturation of the nitrifying community as the main tool of inorganic nitrogen control [7, 9]. In agreement, the established AOB community appreciatively maintained TAN levels throughout the remainder of the study starting from the 8th day.

On the 13th day, the removal of solids coincided with a decrease in NO_2-N levels. This reduction can be attributed to a decrease in organic matter load and the anaerobic patches, which subsequently resulted in a lower denitrification rate [7, 40]. However, it is likely that the subsequent increase in NO_2-N levels was due to the removal of numerous floc-associated slow-growing nitrite-oxidizing bacteria when performing solids removal [2, 5, 7].

Floc volume can vary depending on various factors, including the maturation stage of the floc, temperature, pH, microbial community, and feeding rate [2]. Removing solids on the 13th day of the study may have unintentionally removed beneficial bacteria that help maintain a balanced microbial community [41]. This created an opportunity for filamentous bacteria, which are known to cause excessive floc formation and compromise water quality in biofloc systems, to thrive and increase floc volume in the subsequent days [42]. It is reported that filamentous bacteria contribute to the development of large, irregular, and open flocs [41].

BFT system rely heavily on nitrogen (N) since it acts as a vital nutrient for bacteria while also presenting a possible toxicity hazard to the cultured species [43]. The inorganic nitrogen species (primarily NH_3-N and NO_2-N) are highly toxic to the shrimp, depending on factors such as exposure duration and water quality (pH, DO, temperature, and salinity) [44, 45]. Frias-Espericueta et al. [46] reported a 12 hr LC_{50} value of 0.7 mg/l NH_3-N for PL12 of *L. vannamei* (22.5 mg/l TAN at 28°C, pH 7.92, Salinity 34 ppt). In the present study, shrimp in different treatments were exposed to approximately 1.5 mg/l TAN for almost 2 days (0.08 mg/l NH_3-N at 32°C, pH 7.9, Salinity 35 ppt). It was eight times less than the reported 12 hr LC_{50} value of NH_3-N for PL12 *L. vannamei* [46]. In another study, about 1% mortality rate was reported for PL30 *L. vannamei* after 4 hr exposure to 18 mg/l TAN (0.54 mg/l NH_3-N at 26°C, pH 8, Salinity 38 ppt) [45]. In addition, a concentration of 3.95 mg/l is reported as the safe level of TAN for the same species (0.16 mg/l NH_3-N at Salinity 35 ppt) [47] which is approximately two times more than the highest levels of TAN observed during the initial days of the current study.

On the other hand, a 96 hr LC_{50} value of 8 mg/l NO_2-N is reported for 0.27 g *L. vannamei* juveniles [48]. Nevertheless, a NO_2-N concentration of 29 mg/l for one week had no apparent effects on *L. vannamei* juveniles survival and growth at 3,800–5,000 PL/m^3 [15]. In addition, it was observed that the same species exposed to NO_2-N levels exceeding 20 mg/l for a period of 14 days demonstrated survival rates exceeding 97% during the nursery phase at a density of 4,050 PL/m^3 [8, 49]. Consistent with these findings, the NO_2-N concentrations in our current study remained consistently below 8 mg/l, except for a maximum of 9.14 mg/l in the G3000 group on the 5th day and 11.03 mg/l in the G5000 and G4000 groups on the 21st day.

Moreover, the concentrations of TAN and NO_2-N were lower than those reported for *L. vannamei* cultured at 1,500–6,000 PL/m^3 during the nursery phase [17]. These imply that TAN and NO_2-N levels recorded during the present experiment were unlikely to have an adverse effect on shrimp growth and survival.

The NO_3-N has the least toxic effect on shrimp compared with TAN and NO_2-N [7]. The peak NO_3-N levels recorded during the present study (16 mg/l) were much lower than the lethal concentration of 100 mg/l reported by Van Rijn et al. [50] and Muir et al. [51]. Therefore, it can be assumed that NO_3-N levels had no significant effect on shrimp growth or survival. Similarly, NO_3-N at concentration of 20 mg/l did not adversely affect *L. vannamei* during the nursery phase [8].

Biofloc formation and accumulation is an important characteristic of the BFT systems [2]. The optimal and the maximum acceptable levels of SS for the growth of *L. vannamei* are suggested as 6–10 and 30 ml/l, respectively [7]. The SS levels of 10–15 ml/l are approximately equal to 400–600 mg/l of TDS concentrations [52]. Higher levels of SS and TSS can retard the growth and survival of shrimp due to clogging gills, depleting DO levels by respiration of dense microbial community degrading the organic matters, and increasing NO_2-N and hydrogen sulfide (H_2S) concentrations [53]. However, in the present study, SS and TSS concentrations in all groups were within the optimal range for *L. vannamei*. In addition, G5000 and G4000 registered higher SS levels than G3000 group as a results of higher stocking density and organic carbon supplementation for the reduction of TAN to <0.5 mg/l during the initial days of the experiment.

The results indicated that shrimp growth was not influenced by stocking density. Shrimp in different groups had statistically similar FBW, WG %, WGR, FCR, and SGR %/day. However, it is reported that shrimp final weight decreased with an increase in stocking density at densities ranging from 1,500 to 17,000 PL/m^3 [4, 17]. Shrimp in the study by Esparza-Leal et al. [4] recorded mean final weights of 0.44 g (3,000 PL/m^3) and 0.35 g (6,000 PL/m^3) after 42 days of

culture, however, shrimp in the current study reached a higher weight of 0.49 g (3000 PL/m^3) and 0.48 g (5000 PL/m^3) after 21 days. The observed variation may be attributed to the difference in water quality parameters, specifically lower than recommended levels [7] of salinity (15.5–16.5 ppt), alkalinity (70–80 mg/l $CaCO_3$), and temperature (23–28°C). Meanwhile, SGR values of approximately 24%/day in the preset study were higher than the rates of 9.2–8.7% (3,000–6,000 PL/m^3) and 14.5% (4,400 PL/m^3) reported by Esparza-Leal et al. [4] and Wasielesky et al. [17], respectively. This can also be attributed to the higher levels of floc volume (17–32 ml/l) which were above the optimum floc volume (6 ml/l) for a mixotrophic biofloc system [7]. High volumes of biofloc can adversely affect shrimp growth performance [2, 7].

In addition, FCR in all tested groups was <0.87 which was lower than 0.95 (4,400 PL/m^3, 35 days) and 1.5 (3,000–6,000 PL/m^3, 42 days) reported by Wasielesky et al. [17] and Esparza-Leal et al. [4], respectively. In comparison to other aquatic species, shrimp aquaculture typically exhibits lower FCR values as a result of various contributing factors including high protein diets [7, 54, 55], lower energy demands [7, 56], and superior digestive efficiency [57]. Furthermore, shrimp can consume microbial biomass in biofloc systems, leading to improved feed utilization and a lower FCR [2, 7, 58]. However, the composition and nutritional quality of microbial biomass can vary depending on factors like feed composition, microbial community structure, culture system management, and environmental conditions [58]. In this sense, the water quality indices observed in the present study were found to be superior to those reported by Wasielesky et al. [17] and Esparza-Leal et al. [4] for a mixotrophic biofloc system, as discussed earlier. This difference in water quality may show a healthier bacterial community that has contributed to the comparatively lower FCR values observed in our study, as noted by Samocha [7].

Another remarkable finding is that Esparza-Leal et al. [4] reported shrimp yields of 1.2–1.8 kg/m^3 which was similar to the present study (1.2–1.7 kg/m^3). This similarity was due to better shrimp survival rates in the noted study (91%, 3,000 PL/m^3 and 88%, 6,000 PL/m^3) compared to those in the current study (80%, 3,000 PL/m^3 and 71%, 5,000 PL/m^3). The higher survival rates observed in the discussed studies are likely the result of high-quality feed, which provides the necessary nutrients and immune-boosting supplements, or the utilization of disease-resistant shrimp strains [3].

Nonetheless, shrimp showed good survival rates in the current study, which was not significantly affected by different stocking densities. These data are in agreement with the previous studies on BFT system testing densities close to those in the present experiment [4, 8, 12]. During the nursery phase, survival is the preferred production factor [59]. Therefore, the nursery stocking densities up to 5,000 PL/m^3 (2,000 PL/m^2) tested in the current study are potentially applicable for the commercial production of *L. vannamei* using the mixotrophic BFT system. Considering previous knowledge [4, 7, 49], it may be inferred that the tested densities could be implemented effectively.

5. Conclusions

The results of the current study highlighted that *L. vannamei* PLs can be successfully reared at intensive densities up to 5,000 PL/m^3 (2,000 PL/m^2) until reaching mean weight of 0.5 g during the nursery phase without remarkable retardation in growth, feed utilization, and survival or water quality. In addition, it was found that a mixotrophic BFT system (heterotrophic/chemotrophic) maintains good water quality in intensive systems during the nursery phase.

Data Availability

The authors confirm that the data supporting the findings of this study are available within the article.

Ethical Approval

This study has followed the guidelines of the Committee of Animal Welfare and Research Ethics, Persian Gulf and Oman Sea Ecological Research Center, Bandar Abbas, Iran.

Disclosure

The Employer's had no involvement in the manuscript writing, editing, approval, or decision to publish.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Authors' Contributions

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Ghasem Mohammadi, Mohammad Seddiq Mortazavi, and Mahmoud Hafezieh. The first draft of the manuscript was written by Ghasem Mohammadi and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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