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Research Article

Effect of Salinity on Nursery Bi-Culture of Pacific White Leg Shrimp (*Litopenaeus vannamei*) and Giant Prawn (*Macrobrachium rosenbergii*) in a Biofloc System

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The current study was carried out to examine the effects of four different experimental salinities ($T_1 = 0‰$, $T_2 = 5‰$, $T_3 = 10‰$, and $T_4 = 15‰$) on growth, water quality, proximate composition, total bacterial (TB), and hemocyte counts of white leg shrimp (*Litopenaeus vannamei*) and giant prawn (*Macrobrachium rosenbergii*) in biofloc based nursery bi-culture system for 6 weeks. A total of 12 cylindrical plastic tanks (125 L) filled up 100 L water for rearing *L. vannamei* and *M. rosenbergii* post-larvae (PLs) at an equal ratio: (50 *L. vannamei*: 50 *M. rosenbergii*). At the end of the experiment, for *L. vannamei*, the significantly higher ($p < 0.05$) growth rate was recorded in T_4 (15‰) compared to the other treatments. For *M. rosenbergii*, a significantly higher ($p < 0.05$) growth rate was recorded in T_2 (5‰) than in other treatments. Similar to growth, the best ($p < 0.05$) feed conversion ratio (FCR) for -*L. vannamei* was found at T_4 (15‰) while it was at T_2 (5‰) for*M. rosenbergii*. Gross return, net profit, and benefit–cost ratio (BCR) analysis revealed higher profit T_4 (15‰) than T_3 (10‰), T_2 (5‰), and T_1 (0‰). TB counts were found to be significantly greater ($p < 0.05$) in T_4 than other treatments. Hemocyte counts for *L. vannamei* were significantly higher ($p < 0.05$) in T_4 (15‰) than T_3 (10‰), T_2 (5‰), and T_1 (0‰) and for *M. rosenbergii* hemocyte was significantly higher ($p < 0.05$) in T_1 (0‰) than T_2 (5 ‰), T_3 (10 ‰), and T_4 (15‰). Therefore, it can be suggested that 15‰ salinity will be the best condition for the nursery bi-culture of white leg shrimp (*L. vannamei*) and giant prawn (*M. rosenbergii*) in the biofloc system.

Keywords: bacterial counts; benefit–cost ratio (BCR); nursery bi-culture in biofloc; total hemocyte counts (THC)

1. Introduction

Salinity is one of the vital ecological factors affecting the growth, survival, and dispersal of many aquatic animals [1–3]. Despite many crustaceans being euryhaline [4], the ideal salinity range for optimum growth, survivability, and production competency differ among species [1, 3, 5–7]. The optimum salinity range affects not only the growth performance of a species but the entire production, biology, and well-being of that species [8]. Thus, continuous efforts are made to find out the ideal/optimum salinity range of farmed aquaculture species to maximize production performance and profit [9]. In order to meet the growing demand of increasing global populations, innovative approaches that

promote resilience, increase profitability, assist conservation and environmental preservation, and enable sustainable aquaculture development must be put into practice [10].

Biofloc technology (BFT), an eco-friendly cultivation method, represents a major technological revolution in aquaculture [11, 12]. BFT reduces the environmental effect of intensive aquaculture by providing little to no water renewal [13]. Moreover, the minimum or zero water exchange in BFT [14] significantly reduces production costs and also reduces the environmental impact (minimizing the discharge/effluents). BFT involves beneficial microbes (bacteria, fungi, protozoa, and also algae, and zooplankton) in a mixture with particulate organic matter [15]. BFT enriches water quality, helps in waste management and helps to minimize disease outbreaks in the case of intensive farming systems [16]. In addition, the microorganisms used in BFT produce bacterial protein from inorganic nitrogen (that is readily utilized as food for the aquaculture species, reduces feeding costs, and lowers the carbon footprints) [17–20]. As a result, the target species grows more quickly and enhances immunity without antibiotics, which reduces the adverse effects on the environment [14, 21–24]. The successful farming with BFT relies on careful species selection, focusing on demandable species that flourish and perform well in this system.

The Pacific white-leg shrimp (*Litopenaeus vannamei*) is a euryhaline species, currently the highest-produced crustacean species globally (53%) [25]. Giant prawn (*Macrobrachium rosenbergii*) contributes only 3% to the global crustacean production but has a premium market price and consumer preference due to its larger size and delicious taste [26]. *L. vannamei* is an indigenous species to the western Pacific coast of Latin America, extending from Peru to Mexico [27] and Asian countries for its potential to grow at different salinity environments with higher stocking densities [28]. Although a marine species (inhabits 20−45‰ salinities), *L. vannamei* can tolerate salinities ranging between 0 and 60‰, and farming is currently practiced in a wide variety of salinity levels [29]. This extensive salinity tolerance ability of shrimp has attracted global shrimp farming intrapreneurs to produce this species at an industrial scale [30, 31]. On the other hand, the giant prawn (*M. rosenbergii*) is the most widely farmed freshwater crustacean species, contributing significantly to the global freshwater aquaculture production [32, 33]. *M. rosenbergii* is a freshwater species that requires brackish water (6−12‰) for larval development but can tolerate up to 20‰ salinity [34–37]. *L. vannamei* is a mid-layer inhabitant, while *M. rosenbergii* is a bottom inhabitant [29, 34]. Due to their utilization of different water layers, there is huge potential to test these two species in a bi-culture system, which has not been thoroughly investigated. Both species constitute their own special attributes as good aquaculture candidates; as such, *L. vannamei* and *M. rosenbergii* can be farmed together (bi-culture) for maximizing production and profit. In achieving this goal, optimum farming conditions must be sought out for the bi-culture of these two commercially important species.

BFT has been found to be an effective approach for intensified production of *L. vannamei* and *M. rosenbergii* production separately [13]. However, this technology can be used for the bi-culture of *L. vannamei* and *M. rosenbergii* to maximize production and profit through the efficient use of different water columns. For the successful implementation of BFT for any species, different parameters must be optimized for the sustainable production of target species, including stocking density, salinity levels, feeding frequency, feeding rate, carbon:nitrogen (C:N) ratio (source of carbohydrate) and probiotic species/type/strain. As *L. vannamei* and *M. rosenbergii* have differences in salinity preferences, in-depth research is required to optimize the salinity levels for maximum production and profit.

Nursery rearing is considered one of the prime steps for the successful farming of different crustacean species to achieve uniform growth, better immunity, higher survival rate, and coping up with climatic or environmental stressors. BFT-based nursery-rearing systems can potentially be more efficient in saving production costs by reducing feed conversion ratio (FCR) and nitrogen metabolite accumulation, improving survival performance, and producing healthy juveniles [38]. Therefore, the aim of this study was to compare the production performance (nursery rearing) of Pacific white leg shrimp (*L. vannamei*) and giant prawn (*M. rosenbergii*) at four different investigational salinity levels (0‰, 5‰, 10‰, and 15‰) as an attempt to optimize the salinity level for maximizing production or profit margins.

2. Materials and Methods

2.1. Acclimation Periods and Species Acquisition. The current study was carried out over a 6 weeks' time frame (from February to April, 2023) at the International Institute of Aquaculture and Aquatic Sciences (I-AQUAS), University Putra Malaysia (UPM). Two round fiber tanks (1000 L) were prepared for the nursery acclimation phase for the post-larvae (PLs) of two target species (*L. vannamei* and *M. rosenbergii*). Prior to the preparation of acclimation tanks, seawater (30‰) and freshwater (0‰) were collected (preserved in two separate water holding tanks: 2000 L each), UV treated, and mixed in equal volumes to prepare 15‰ water. Then, two round fiber tanks (1000 L each) were filled with 15‰ water for acclimating *M. rosenbergii* and *L. vannamei*. Three air stones were placed in each acclimation tank to vigorously aerate the water. In total, 1200 individuals of *L. vannamei* (PL_{10}) and 1200 *M. rosenbergii* (PL_{10}) were collected from KG Acheh Setiawan Peraka and Hilex Aquatic Sdn Bhd, Jeram Kuala Selangor, respectively. *L. vannamei* and *M. rosenbergii* were stocked and maintained in two the separate tanks (1000 L round fiber tanks) containing 15‰ water for 4 days of acclimation. A commercial pellet feed (40% crude protein, miniscule crumbs: 0.25–1 mm in size) was given at the rate of 75% body weight to the PLs three times daily (at 09:00, 14:00, and 18:00).

2.2. Starter Preparation. Two probiotic strains (*Lysinibacillus fusiformis* SPS11, *Enterococcus hirae* LAB3) were collected from the Laboratory of Fish Health, Department of Aquaculture, Faculty of Agriculture, UPM in this experiment for using in the biofloc system. The biofloc starter was prepared

Table 1: Description of four different salinity-based biofloc systems used for the experiment of shrimp and prawn PLs.

Experimental groups	Descriptions	Stocking ratio	Shrimp/prawn PLs (PLs × replicate)
T_1 (0‰)	Freshwater (0‰) based biofloc system	50*V*:50*M*	$(V=50+M=50)\times 3=300$
T_2 (5‰)	Low salinity (5‰) based biofloc system		$(V=50+M=50)\times 3=300$
T_3 (10‰)	Medium salinity (10‰) based biofloc system		$(V=50+M=50)\times 3=300$
T_4 (15‰)	Medium-high salinity (15‰) based biofloc		$(V=50+M=50)\times 3=300$

Note: M, prawn (*M. rosenbergii*); *V*, shrimp (*L. vannamei*).
Abbreviation: PLs, post-larvae.

by mixing 55 mL molasses (as carbohydrate source), 1 L probiotics consortium (5×10^8 CFU mL^{-1}) of *L. fusiformis* SPS11, and *E. hirae* LAB3 in 5.50 L of 15‰ water in a plastic bucket [39, 40]. The entire mixture was covered with the aluminum foil paper (in an aerobic condition) to keep it free from contamination and maintained at ambient temperature for 6 days for fermentation (a sweet smell indicated the growth of target probiotic bacteria). This fermented starter was used as the stock or mother solution for experimental purposes; added immediately (within 1 h) in the 12 experimental tanks containing water of four different salinities (0‰, 5‰, 10‰, and 15‰).

2.3. Experimental Design and Procedure (Tank Setting). A total of 12 cylindrical plastic tanks (125 L each) were cleaned and prepared for this experiment: bi-culture for the nursery rearing of white leg shrimp (*L. vannamei*) and giant prawn (*M. rosenbergii*) in replicated tanks (Table 1) for 6 weeks. Initially, the 12 tanks were filled up to 100 L with 15‰ water, and an equal number of experimental animals (100/tank: 50 *L. vannamei* + 50 *M. rosenbergii*) were randomly allocated in each tank. Following this stocking in experimental tanks, attempts were made to achieve four different salinity levels ($T_1=0$‰, $T_2=5$‰, $T_3=10$‰, and $T_4=15$‰) with three replicated tanks per salinity. Salinities were reduced by adding freshwater (dechlorinated tap water) in the tanks. Salinity was reduced by 2.50‰ per day by adding freshwater in the tanks to achieve the target salinities. It took 6 days to achieve 0‰, 4 days to achieve 5‰, and 2 days for 10‰. Therefore, salinity reduction started 4 days early for T_1 (0‰) and 2 days early for T_2 (5‰) compared to T_3 (10‰) to achieve the target treatment salinities simultaneously. Then, a 546.25 mL starter was added in each experimental tank. The floc concentration was found in the range of 0.50–1mL/L after 4 days of adding the starter, which is suitable for stocking PL [17]. In parallel, shrimp and prawn PLs were challenged with gradual salinity reduction in separate tanks to finally transfer them to the experimental tanks containing bioflocs. Immediately after achieving the target salinities, 100 PLs (50 *L. vannamei* + 50 *M. rosenbergii*) were stocked in each experimental tank containing bioflocs to maintain the stocking density of 1 PL per L [41]. *L. vannamei* and *M. rosenbergii* PLs were maintained in the replicated experimental tanks ($T_1=0$‰, $T_2=5$‰, $T_3=10$‰, and $T_4=15$‰) for 6 weeks to compare the production performance. These salinities were chosen because both of these two species are able to tolerate this salinity range [42–47]. All the experimental tanks were maintained with continuous aeration using air stones (connected to an aerator): one air stone for each tank. The initial body weight of *L. vannamei* was 23.4 ± 0.5 mg, and for *M. rosenbergii* 40.10 ± 0.35 mg during stocking in the experimental tanks.

2.4. Feed Application. A commercial shrimp feed manufactured by CRP Dindings (40% crude protein, 5% fat, 4% crude fiber, and 12% moisture) was given to the experimental *L. vannamei* and *M. rosenbergii* PLs four times daily (08:00, 12:00, 03:00, and 06:00). Initially, feed was given at the rate of 20% of total biomass, which was gradually adjusted to 10% according to the methods outlined in [38, 48].

2.5. Carbon Source Used in the Experimental Tanks. Molasses was added daily in the experimental tanks to keep the nitrogen-to-carbon ratio at 10:1 [49]. The contents of the feed (CRP Dindings) were 90% of dry matter (moisture = 10%) and 30% digestibility. Normally, every feed contains 50% carbon when it is formulated [50, 51]. The daily amount of C:N ratio was calculated according to the feed supply's overall protein level. Molasses was mixed with 50 mL of water and kept at ambient temperature overnight in an anaerobic environment. By multiplying the quantity of carbon by 0.31 (molasses contains 31.88% organic carbon, according to the test results of Material Characterization Laboratory, UPM), the daily amount of molasses was estimated as $T_1=0.89$ ($[T_1R_1=0.29]+[T_1R_2=0.28]+[T_1R_3=0.32]$), $T_2=1.00$ ($[T_2R_1=0.33]+[T_2R_2=0.35]+[T_2R_3=0.32]$), $T_3=0.96$ ($[T_3R_1=0.32]+[T_3R_2=0.31]+[T_3R_3=0.33]$), and $T_4=1.16$ ($[T_4R_1=0.38]+[T_4R_2=0.38]+[T_4R_3=0.40]$). In the morning (10:00 a.m.), molasses was poured into 12 biofloc tanks according to the above-mentioned amount.

2.6. Measuring Water Quality Parameters. Water pH and temperature were measured daily at 09:00 h using a Digital pH Meter (YIERYI 3 In 1 PH Tester, Yieryi Tools, China). Ammonia (NH_3/NH_4^+), nitrate (NO_3^-), and nitrite (NO_2) were measured every day at 10:30 h using an API test kit (MARS Fishcare North America). Dissolved oxygen (DO) (ppm) was measured once in a week at 08:30 h using a Doc SMART SENSOR Digital Dissolved Oxygen Meter. Bioflocs volume (BFV) was assessed with the support of Imhoff cones (after 30 min precipitation of 1 L of water samples) every day according to the methods of [52].

2.7. Concentration of Microorganisms in the Biofloc. At the end of this experimentation, total beneficial/probiotic bacterial loads were calculated. Tryptic soya agar (TSA; Difco,

Detroit, MI, USA) was used for the estimation of total bacterial (TB) loads with the dilution factors of 10^6, 10^7, and 10^8. From each tank, 10 mL water samples were collected to calculate the TB counts according to the methods outlined in [11, 53]. Finally, the TB loads were estimated by the following equation:

$$\text{Total bacteria}(\text{CFU/mL}) = \frac{\text{No of colony} \times \text{dilution factor}}{\text{volume of the inoculants}}. \quad (1)$$

2.8. Total Hemocyte Counts (THC). At the end of this experiment, five individuals of *L. vannamei* and five *M. rosenbergii* were randomly collected from each tank. In total, 120 individuals were used for THC; 30 replicated samples for each experimental salinity. Live specimens were disinfected by soaking in 70% ethanol to prevent any contamination. From each species, 50 µL of hemolymph sample was collected by micro-injection inserted through the ventral sinus of the first abdominal segment [54]. Each syringe was prefilled with 100 µL of anticoagulants solution (10 mM EDTA [ethylenediaminetetraacetic acid], 30 mM trisodium citrate, 340 mM sodium chloride, at pH 7.55, with osmolality adjusted to 718 mOsm/kg by the addition of 115 mM glucose) to prevent clotting of hemolymph samples [54]. The anticoagulated hemolymph sample was shifted into a Neubauer Hemocytometer (Loptik Labor, Germany) for inspection under a light microscope (BX43, OLYMPUS) to count the number of total hemocyte cells. The Neubauer chamber has a volume of 0.0025 mm^3 and a depth of 0.10 mm. To calculate the average value, the THC from two Neubauer chamber partitions was divided into two equal parts [55]. Five compartments were counted from every segment to represent the entire part. THC were finally counted by the following formula:

$$\text{THC}\,(\text{cell/mL}) = \frac{\text{Number of cells} \times 10,000}{\text{Number of square} \times \text{dilition factor}}. \quad (2)$$

2.9. Proximate Composition Analysis. At the end of experimental trials, biofloc samples were collected from each tank by using a plankton net (mesh size 60 µm) while *L. vannamei* and *M. rosenbergii* samples were collected by scope net for proximate composition analysis. After reaching a consistent weight, the biofloc, *L. vannamei* and *M. rosenbergii* samples were dried in an oven at 55°C and stored at −20°C for further analysis. Proximate compositions of *L. vannamei*, *M. rosenbergii*, and bioflocs were determined in triplicate using the AOAC standard procedures [56]. In brief, the amount of ash was determined by heating the samples at 600°C for 5 h. The lipid contents of the samples were ascertained by using a lipid analyzer (Foss Tecator, SoxtecTM8000). For determining the protein levels, samples underwent acid digestion for 60 min, following which protein levels were quantified Kjeldal method [56]. Finally, the following formula was used to determine the total amount of carbohydrates [57]:

$$\begin{aligned}\text{Carbohydrate}\,(\%\text{DW}) = 100 - [&\text{crude protein}\,(\%\text{DW}) \\ &+ \text{lipid}\,(\%\text{DW}) + \text{ash}\,(\%\text{DW})].\end{aligned} \quad (3)$$

2.10. Growth Parameters and Economic Analysis. From each tank, 10 individuals of *L. vannamei* and *M. rosenbergii* samples were randomly collected (30 individuals per treatment for each species) once a week to measure the growth performance. Survival rates of *L. vannamei* and *M. rosenbergii* samples were calculated by counting the total number of survivors at the end of this study (after 42 days of rearing). The final weight, weight gain, FCR, specific growth rate (SGR), and survival rate were calculated using the following formula [58]:

$$\text{Specific growth rate}\,(\text{SGR}, \%/\text{day}) = \frac{\ln W_f - \ln W_i}{t} \times 100, \quad (4)$$

$$\text{Weight gain}\,(\%) = \frac{\text{Mean final weight} - \text{Mean initial weight}}{\text{Mean initial weight}} \times 100, \quad (5)$$

$$\text{Daily weight gain}\,(\text{DWG})\,(\text{mg/day}) = \frac{W_f - W_i}{t}, \quad (6)$$

$$\begin{aligned}\text{Biomass gain}\,(\text{mg}) = &\text{Harvested biomass}\,(\text{mg}) \\ &- \text{Stocked biomass}\,(\text{mg}),\end{aligned} \quad (7)$$

$$\text{Feed conversion ratio} = \frac{\text{Total amount feed given}\,(\text{mg})}{\text{Weight gain}\,(\text{mg})}, \quad (8)$$

$$\text{Survival}\,(\%) = \frac{\text{Number of harvested individuals}}{\text{Number of stocked individuals}} \times 100, \quad (9)$$

$$\text{Benefit-cost ratio}\,(\text{BCR}) = \frac{\text{Total net return}}{\text{Total cost}}, \quad (10)$$

where W_f is final weight (mg), W_i is initial weight (mg), and t is culture duration (day).

2.11. Statistical Analysis. Initially, raw data (body weight, proximate composition, survivability, hemocyte counts, etc.) were loaded in Microsoft Excel 2011. Different types of statistical analyses were carried out with SPSS (version 25) software. The homogeneity and normality of the variances were assessed using the Shapiro–Wilks and Levene's tests. One way analysis of variance (ANOVA) tests was used to investigate the variations between the experimental salinities. The post hoc assessment of the mean across distinct salinity groups was done using Duncan's multiple range tests, while significant variations were found at the 5% level of significance ($p < 0.05$).

TABLE 2: Different aspects of water quality parameters in the biofloc-based nursery bi-culture systems under four different salinities for 42 days (mean of six samplings).

Parameters	T_1 (0‰)	T_2 (5‰)	T_3 (10‰)	T_4 (15‰)	*p*-Value
pH (ppm)	7.70 ± 0.02^a	7.40 ± 0.25^c	7.55 ± 0.05^b	7.59 ± 0.06^b	0.001
Ammonia (ppm)	0.20 ± 0.1^a	0.19 ± 0.08^a	0.35 ± 0.16^b	0.45 ± 0.17^c	0.001
Temperature (°C)	27.43 ± 0.07^a	27.42 ± 0.05^a	27.39 ± 0.04^b	27.44 ± 0.07^a	0.003
DO (ppm)	7.10 ± 0.23^c	7.33 ± 0.17^b	7.49 ± 0.16^a	7.28 ± 0.23^b	0.001
Nitrate (ppm)	2.84 ± 0.72^a	2.41 ± 0.55^b	3.12 ± 0.82^c	4.38 ± 0.85^d	0.001
Nitrite (ppm)	0.05 ± 0.01^a	0.03 ± 0.01^a	0.11 ± 0.03^b	0.13 ± 0.05^b	0.001
Floc volume (mL/L)	5.67 ± 2.37^a	5.74 ± 0.40^a	8.11 ± 3.06^a	8.02 ± 0.97^a	0.001

Note: Data present at means ± standard deviation. The different letters in the same row indicate significant differences at $p < 0.05$.
Abbreviation: DO, dissolved oxygen.

Every data point in the tables was shown as mean ± SD (standard deviation), with a significant difference at $\alpha = 5\%$.

3. Results

3.1. Water Quality Parameters. Table 2 and Figure 1 indicate the water quality parameters recorded in this study. Among the tested parameters, pH (ppm) and ammonia (NH_3/NH_4^+) (ppm), temperature (°C), DO (ppm), nitrate (NO_{3^-}) (ppm), nitrite (NO_{2^-}) (ppm), and floc volume (mL/L) levels varied significantly ($p < 0.05$) between the four salinities.

3.2. Total Microbial Loads in the Biofloc. Experimental salinity treatments significantly affected ($p < 0.05$) the microbial loads ($12.05–132.67 \times 10^9$ CFU/mL) in the biofloc system (Table 3). An increasing trend was observed for the total microbial loads with increasing salinity and ranked as T_1 ($0‰ = 12.05 \pm 0.93 \times 10^9$) < T_2 ($5‰ = 38.32 \pm 7.04 \times 10^9$) < T_3 ($10‰ = 59.77 \pm 4.90 \times 10^9$) < T_4 ($15‰ = 132.67 \pm 21.14 \times 10^9$) (Table 3).

3.3. THC of the Two Species. Experimental salinity treatments significantly affected ($p < 0.05$) the THC (cells/mL) of the Pacific whiteleg shrimp ($1.48–3.10 \times 10^5$) and giant prawn ($3.99–1.53 \times 10^5$) in the biofloc system (Table 4). For *L. vannamei* an increasing trend was observed for the total hemocyte with increasing salinity and ranked as T_1 ($0‰ = 1.48 \times 10^5$) < T_2 ($5‰ = 2.50 \times 10^5$) < T_3 ($10‰ = 2.68 \times 10^5$) < T_4 ($15‰ = 3.10 \times 10^5$) (Table 4) but for *M. rosenbergii* decreasing trend was observed for the total hemocyte with increasing salinity and ranked as T_1 ($0‰ = 3.99 \times 10^5$) > T_2 ($5‰ = 3.54 \times 10^5$) > T_3 ($10‰ = 2.76 \times 10^5$) > T_4 ($15‰ = 1.53 \times 10^5$) (Table 4).

3.4. Proximate Composition Analysis. Within different salinities, there were significant differences ($p < 0.05$) in the crude protein, lipid, ash, and carbohydrate contents of the *L. vannamei* and *M. rosenbergii* muscle (Table 5). For *L. Vannamei*, significantly ($p < 0.05$) higher levels of crude protein, lipid, and ash were found in T_4 (15‰) compared to T_1 (0‰), T_2 (5‰), and T_3 (10‰) (Table 5). The highest level of carbohydrate ($p < 0.05$) was found in T_1 (0‰) compared to T_2 (5‰), T_3 (10‰), and T_4 (15‰) (Table 5). For *M. rosenbergii*, significantly ($p < 0.05$) higher levels of crude protein and lipid were found in T_4 (15‰) compared to T_1 (0‰), T_2 (5‰), and T_3 (10‰) (Table 5). Moreover, the highest levels ($p < 0.05$) of ash and carbohydrate were found in T_1 (0‰) compared to T_2 (5‰), T_3 (10‰), and T_4 (15‰) (Table 5).

3.5. Proximate Composition of Biofloc. Table 6 demonstrates that the crude protein, fat, ash, and carbohydrate content of the biofloc varied significantly ($p < 0.05$) depending on the salinity levels. A decreasing trend was observed for the crude protein, lipid, and ash with increasing salinity and ranked as T_1 (0‰) > T_2 (5‰) > T_3 (10‰) > T_4 (15‰) but vice versa for carbohydrates (Table 6).

3.6. Growth Parameters. Growth performance and economic analysis of *L. vannamei* and *M. rosenbergii* raised in four different salinities were presented in Tables 7 and 8 and Figure 2. Survival rate (%) of *L. vannamei* was found to be significantly higher ($p < 0.05$) in T_4 (15‰) (75.33 ± 5.03) than T_3 (10‰) (44.67 ± 3.61), T_2 (5‰) (26.67 ± 1.61), and T_1 (0‰) (24.00 ± 2.00). Significantly higher ($p < 0.05$) survival rate (%) of *M. rosenbergii* was found in T_2 (5‰) (93.33 ± 1.15) compared to T_3 (10‰) (92.00 ± 3.46), T_4 (15‰) (88.67 ± 7.57) and T_1 (0‰) (87.33 ± 6.43).

FCR for *L. vannamei* was found to be noticeably ($p < 0.05$) better in T_4 (15‰) (1.21) than T_1 (0‰) (2.59). For *M. rosenbergii*, significantly better FCR ($p < 0.05$) was found in T_2 (5‰) (1.12) compared to T_1 (0‰) (1.36). SGR (%/day) for *L. vannamei* was significantly higher ($p < 0.05$) in T_4 (15‰) (6.79) than T_1 (0‰) (5.86). In contrast, significantly higher ($p < 0.05$) SGR (%/day) was observed for *M. rosenbergii* in T_1 (0‰). Weight gain (%) for *L. vannamei* was significantly higher ($p < 0.05$) in T_4 (15‰) (1632.26 ± 10.40) compared to T_3 (10‰) (1187.57 ± 279.11), T_2 (5‰) (1135.71 ± 315.70), and T_1 (0‰) (1076.66 ± 105.72). Daily weight gain (mg/day) for *L. vannamei* was significantly higher ($p < 0.05$) in T_4 (15‰) (9.20 ± 0.14) than T_3 (10‰) (6.67 ± 1.46), T_2 (5‰) (6.34 ± 1.74) and T_1 (0‰) (6.00 ± 0.59). Weight gain (%) and daily weight gain (mg/day) for *M. rosenbergii* were significantly higher ($p < 0.05$) in T_2 (5‰).

Biomass gain (mg) for *L. vannamei* was significantly higher ($p < 0.05$) in T_4 (15‰) (386.27 ± 5.75) over T_3 (10‰) (280.20 ± 61.38), T_2 (5‰) (266.27 ± 73.00), and T_1 (0‰) (251.93 ± 24.74). Biomass gain (mg) for *M. rosenbergii* was recorded as significantly higher ($p < 0.05$) in T_2 (5‰)

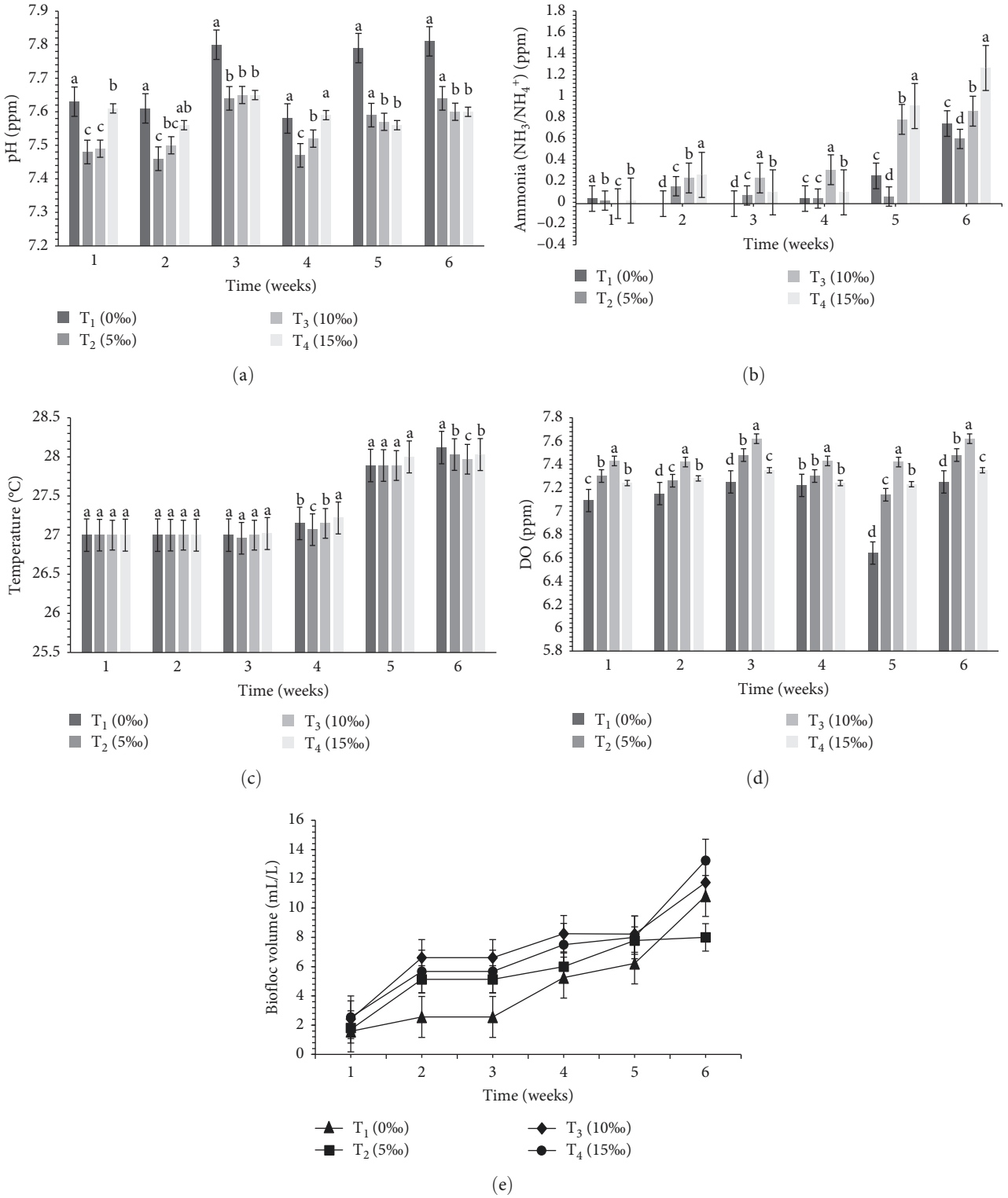

FIGURE 1: Effect of salinities on water quality parameters (weekly variations in different water quality parameters): (a) pH (ppm), (b) ammonia (NH_3/NH_4^+) (ppm), (c) temperature (°C), (d) dissolve oxygen (DO), and (e) biofloc volume (mL/L). *Vertical bar* indicates standard deviation (SD).

than other treatments. Total production (g) both (*L. vannamei* + *M. rosenbergii*) was significantly higher ($p < 0.05$) in T_4 (15‰) (75.90 g) than T_3 (10‰) (52.94 g), T_2 (5‰) (50.44 g), and T_1 (0‰) (36.60 g).

3.7. Economic Analysis. The higher ($p < 0.05$) gross return, net profit, and benefit–cost ratio (BCR) were found in T_4 (15‰) (1.87) and followed by T_3 (10‰) (1.22), T_2 (5‰) (0.80), and T_1 (0‰) (0.72), respectively (Table 8).

Table 3: Total bacterial loads (10^9 CFU/mL) in four different salinities using biofloc systems for rearing *L. vannamei* and *M. rosenbergii* post larvae.

Parameter	T_1 (0‰)	T_2 (5‰)	T_3 (10‰)	T_4 (15‰)	*p*-Value
Total bacteria	12.05 ± 0.93^{d}	38.32 ± 7.04^{c}	59.77 ± 4.90^{b}	132.67 ± 21.14^{a}	0.001

Note: Data present at means ± standard deviation. The different letters in the same row indicate significant differences at $p < 0.05$.

Table 4: Total hemocyte counts (10^5 cells/mL) of *L. vannamei* and *M. rosenbergii* post-larvae under four different salinities in the biofloc system.

Species	T_1 (0‰)	T_2 (5‰)	T_3 (10‰)	T_4 (15‰)	*p*-Value
L. vannamei	1.48 ± 0.14^{d}	2.50 ± 0.11^{c}	2.68 ± 0.15^{b}	3.10 ± 0.12^{a}	0.001
M. rosenbergii	3.99 ± 0.08^{a}	3.54 ± 0.03^{b}	2.76 ± 0.17^{c}	1.53 ± 0.06^{d}	0.001

Note: Data present at means ± standard deviation. The different letters in the same row indicate significant differences at $p < 0.05$.

Table 5: Proximate composition (% dry weight) of *L. vannamei* and *M. rosenbergii* post-larvae under four different salinities in the biofloc system.

Species	Parameters	T_1 (0‰)	T_2 (5‰)	T_3 (10‰)	T_4 (15‰)	*p*-Value
L. vannamei	Protein	64.14 ± 0.15^{d}	$66.75 \pm .0.12^{c}$	66.80 ± 0.09^{b}	68.56 ± 1.09^{a}	0.001
	Lipid	2.96 ± 0.06^{d}	4.37 ± 0.07^{c}	5.21 ± 0.04^{b}	6.36 ± 0.08^{a}	0.001
	Ash	17.25 ± 0.80^{d}	17.42 ± 0.70^{b}	17.56 ± 0.65^{c}	17.64 ± 0.95^{a}	0.001
	Carbohydrate	15.65 ± 0.50^{a}	11.46 ± 0.65^{b}	10.43 ± 0.80^{c}	7.44 ± 0.65^{d}	0.001
M. rosenbergii	Protein	63.20 ± 0.04^{c}	65.27 ± 0.09^{b}	65.75 ± 0.05^{a}	65.76 ± 0.08^{a}	0.001
	Lipid	3.76 ± 0.03^{d}	$4.10 \pm .05^{c}$	4.57 ± 0.07^{b}	4.95 ± 0.08^{a}	0.001
	Ash	15.92 ± 0.85^{a}	15.63 ± 0.90^{b}	15.55 ± 0.85^{c}	15.50 ± 0.50^{d}	0.001
	Carbohydrate	17.12 ± 0.75^{a}	15.00 ± 0.45^{b}	14.13 ± 0.25^{b}	13.79 ± 0.70^{b}	0.001

Note: Data present at means ± standard deviation. The different letters in the same row indicate significant differences at $p < 0.05$.

Table 6: Proximate composition (% dry weight) of floc in a bi-culture system of *L. vannamei* and *M. rosenbergii* post-larvae under different salinities in the biofloc system.

Parameters	T_1 (0‰)	T_2 (5‰)	T_3 (10‰)	T_4 (15‰)	*p*-Value
Protein	24.90 ± 0.73^{a}	23.96 ± 0.83^{b}	23.95 ± 0.80^{b}	17.70 ± 0.96^{c}	0.001
Lipid	$0.60 \pm .03^{a}$	0.26 ± 0.01^{b}	0.22 ± 0.02^{ab}	0.15 ± 0.01^{b}	0.001
Ash	23.60 ± 0.85^{a}	23.55 ± 0.70^{b}	22.45 ± 0.90^{c}	22.40 ± 0.30^{d}	0.001
Carbohydrate	50.90 ± 0.25^{a}	52.23 ± 0.75^{b}	53.38 ± 0.70^{c}	59.75 ± 0.90^{d}	0.001

Note: Data present at means ± standard deviation. The different letters in the same row indicate significant differences at $p < 0.05$.

This financial evaluation did not take into account the lab equipment that provided by I-AQUAS or the Department of Aquaculture, Universiti Putra Malaysia.

4. Discussion

4.1. Water Quality Parameters. BFT is known to improve water quality by reducing nitrogenous waste [59] and minimizing excessive water use and environmental concerns through reduced effluent discharge [60, 61]. The efficiency of BFT depends on optimizing different parameters for target species, of which C:N ratio is very crucial. Earlier investigations indicate that the optimum range of different water quality parameters for different crustaceans includes: DO = 5–8 ppm, pH = 6.80–8.20, ammonia = 0.06–0.20 ppm, temperature = 26 −30°C, nitrate = 0.30–1.20 ppm, nitrite = 0.02–0.10 ppm, and floc concentration (mL/L) = 6–80 mL/L [24, 36, 62–72]. In this study, all of these water quality parameters (DO, temperature, pH, ammonia, nitrate, nitrite, and floc concentration) were found to be within the optimum range (Table 2) in the experimental tanks, clearly indicate the important functions of BFT in maintaining water quality within the optimum range for the study species. In the current study, ammonia, nitrate, and nitrite were significantly higher ($p < 0.05$) in T_4 (15‰) (Table 2) that were similar to the findings of earlier studies [58, 67].

4.2. Biofloc Microorganisms. The presence of a beneficial microbial community in BFT is known to enhance feed utilization and animal growth by providing feed supplements and improving water quality via detoxifying excessive nutrients [73]. Abundance of the microbial protein in the BFT contains heterotrophic bacteria that serve as a nutritional source, improve immunity and also help to reduce the loads

Table 7: Growth parameters of shrimp and prawn post-larvae under four different salinities in the biofloc system after 42 days rearing.

Parameters	T_1 (0‰)	T_2 (5‰)	T_3 (10‰)	T_4 (15‰)	*p*-Value
Initial length (mm)					
V	13.10 ± 0.1^{b}	12.93 ± 0.23^{c}	13.10 ± 0.10^{b}	13.17 ± 0.06^{a}	0.001
M	14.70 ± 0.10^{c}	14.77 ± 0.12^{a}	14.73 ± 0.06^{b}	14.63 ± 0.12^{d}	0.001
Initial weight (mg)					
V	23.40 ± 0.20^{b}	23.47 ± 0.23^{b}	23.67 ± 0.50^{a}	23.67 ± 0.50^{a}	0.001
M	40.20 ± 0.20^{b}	40.40 ± 0.35^{a}	40.27 ± 0.12^{ab}	40.10 ± 0.26^{b}	0.017
Final length (mm)					
V	31.73 ± 4.15^{c}	34.47 ± 5.75^{b}	35.00 ± 3.61^{b}	40.07 ± 0.90^{a}	0.001
M	29.00 ± 1.97^{a}	28.47 ± 2.86^{ab}	27.80 ± 3.33^{b}	28.33 ± 1.50^{ab}	0.112
Final weight (mg)					
V	289.20 ± 42.91^{c}	289.53 ± 73.24^{c}	303.87 ± 60.93^{b}	409.93 ± 6.25^{a}	0.001
M	199.93 ± 4.69^{d}	277.60 ± 93.58^{a}	229.47 ± 76.37^{b}	222.40 ± 44.55^{c}	0.001
Survival rate (%)					
V	24.00 ± 10.00^{d}	26.67 ± 13.61^{c}	44.67 ± 13.61^{b}	75.33 ± 5.03^{a}	0.001
M	87.33 ± 6.43^{d}	93.33 ± 1.15^{a}	92.00 ± 3.46^{b}	88.67 ± 7.57^{c}	0.001
FCR					
V	2.59^{d}	2.21^{c}	1.40^{b}	1.21^{a}	0.001
M	1.36^{d}	1.12^{a}	1.28^{b}	1.34^{c}	0.001
SGR, (%/day)					
V	5.86^{d}	5.92^{c}	6.04^{b}	6.79^{a}	0.001
M	4.73^{a}	4.50^{b}	4.05^{c}	4.04^{c}	0.001
Weight gain (%)					
V	1076.66 ± 105.72^{d}	1135.71 ± 315.70^{c}	1187.57 ± 279.11^{b}	1632.26 ± 10.40^{a}	0.001
M	397.34 ± 12.97^{d}	585.86 ± 224.74^{a}	470.13 ± 190.81^{b}	454.15 ± 107.94^{c}	0.001
DWG (mg/day)					
V	6.00 ± 0.59^{b}	6.34 ± 1.74^{b}	6.67 ± 1.46^{b}	9.20 ± 0.14^{a}	0.001
M	3.80 ± 0.13^{d}	5.65 ± 2.22^{a}	4.50 ± 1.82^{b}	4.34 ± 1.05^{c}	0.001
Biomass gain (mg)					
V	251.93 ± 24.74^{d}	266.27 ± 73.00^{c}	280.20 ± 61.38^{b}	386.27 ± 5.75^{a}	0.001
M	159.73 ± 5.41^{d}	237.20 ± 93.24^{a}	189.20 ± 76.45^{b}	182.30 ± 44.29^{c}	0.001
Production (g)					
V	10.41 ± 0.30^{d}	11.58 ± 0.95^{c}	21.27 ± 1.34^{b}	46.32 ± 0.28^{a}	0.001
M	26.19 ± 0.24^{d}	38.86 ± 4.40^{a}	31.67 ± 3.51^{b}	29.58 ± 9.79^{c}	0.001
Total production (g)	36.60^{d}	50.44^{c}	52.94^{b}	75.90^{a}	0.001

Note: Data present at means ± standard deviation. The different letters in the same row indicate significant differences at $p < 0.05$. *M*, prawn (*M. rosenbergii*); *V*, shrimp (*L. vannamei*).
Abbreviations: DWG, daily weight gain; FCR, feed conversion ratio; SGR, specific growth rate.

Table 8: Economic analysis for the bi-culture of *L. vannamei* and *M. rosenbergii* post-larvae under four different salinities in the biofloc system after 42 days of rearing.

Parameters	T_1 (0‰)	T_2 (5‰)	T_3 (10‰)	T_4 (15‰)	*p*-Value
Total cost (USD)	5.85^{b}	5.86^{b}	5.86^{b}	5.88^{a}	0.035
Gross return (USD)	10.08^{d}	10.55^{c}	13.02^{b}	16.91^{a}	0.001
Net profits (USD)	4.25^{d}	4.69^{c}	7.16^{b}	11.02^{a}	0.001
BCR	0.72^{c}	0.80^{c}	1.22^{b}	1.87^{a}	0.001

Note: The different letters in the same row indicate significant differences at $p < 0.05$. PL rate 0.029 USD, feed 0.924 USD/kg, and selling price 0.263 USD/*L. vannamei* and 0.158 USD/*M. rosenbergii*. 1 RM = 0.21 USD.
Abbreviation: BCR, benefit–cost ratio.

of harmful microbes [19, 62, 74–76]. Moreover, the heterotrophic bacteria in the BFT control the presence of ammonia and other nitrogenous metabolites and convert wastes into additional food sources for the rearing of target species [71, 77]. Some earlier investigations found that even at a higher stocking density of target crustacean species, *Vibrio* load was lower in the biofloc system compared to the conventional system while exhibited a higher abundance of

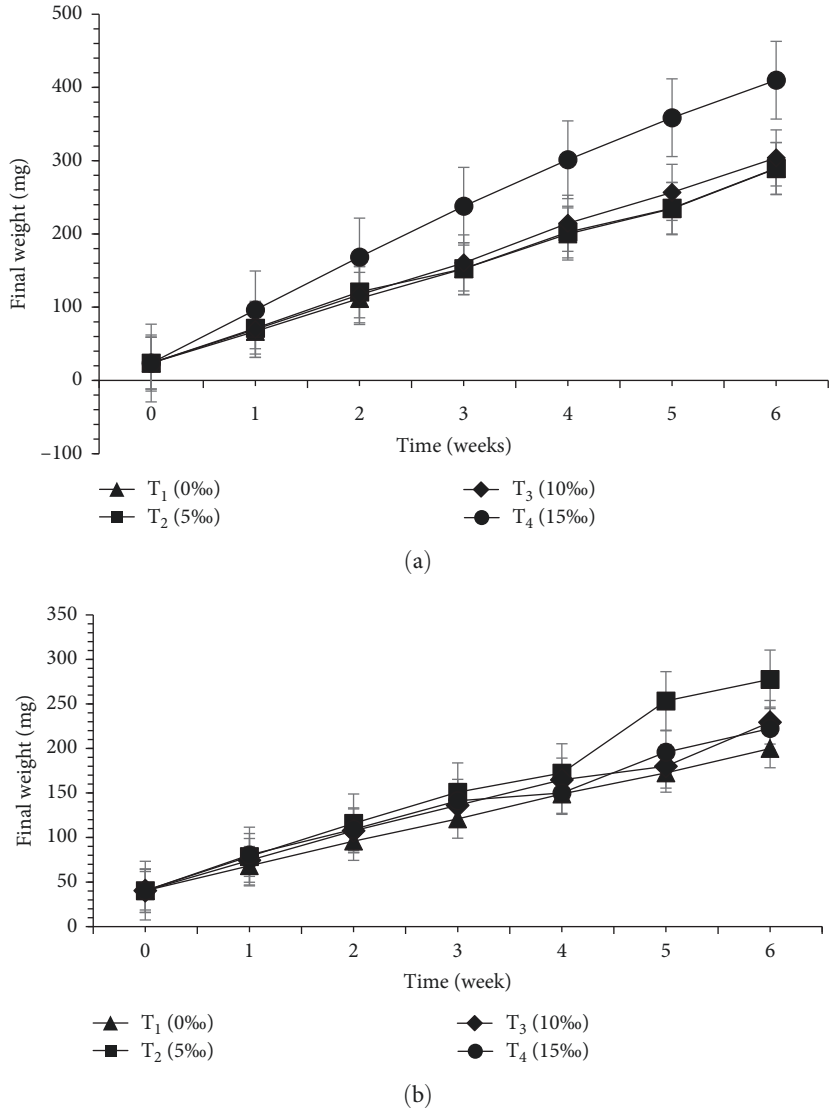

FIGURE 2: Effect of salinities on the final weight of (a) shrimp (*L. vannamei*) and (b) prawn (*M. rosenbergii*) post-larvae reared in the biofloc systems. *Vertical bar* indicates standard deviation (SD).

beneficial bacteria, including *Bacteroidia*, *Bacteroidales*, *Flavobacteriia*, *Flavobacteriales*, and *Mollicutes* [78–80]. In the current study, TB load (10^9CFU/mL) was significantly higher ($p<0.05$) in T_4 (15‰) (132.67 ± 21.14) than T_3 (10‰) (59.77 ± 4.90), T_2 (5‰) (38.32 ± 7.04), and T_1 (0‰) (12.05 ± 0.93) (Table 3), which is likely due to these bacteria being more brackish/marine [81]. Salinity is a primary determinant of the growth of heterotrophic bacteria and the nitrification processes [82, 83]. In the current study, the highest ($p<0.05$) amount of ammonia, nitrite, and nitrate was found in T_4 (15‰), indicating a higher intensity of nitrification processes, which was similar to an earlier investigation [58]. The heterotrophic and nitrifying bacteria appear to be more active in higher salinities [84]. This suggested that more bacterial loads were found at T_4 (15‰) due to increased salinity. According to Hosain et al. [67], the highest abundance of TB load was observed at 15‰ when only the giant prawn (*M. rosenbergii*) PLs reared at four different salinities (0‰, 5‰, 10‰, and 15‰). Another study on *O. niloticus* revealed changes in the abundance of TB loads when grown at varying salinities (0‰, 10‰, or 20‰) [85].

4.3. THC of the Two Species. Hemocyte counts generally indicate the immunity status of crustaceans; higher levels of hemocyte counts represent better immunity, while lower counts represent poor immunity [8, 86, 87]. Therefore, the amount/number of circulatory hemocyte cells has been recognized as an effective immunological index [88]. Crustacean hemocyte cells consist of hyaline, semi-granular, and big granular cells that participate in cellular immune reactions (such as phagocytosis), which is the main mechanism for fighting against or killing germs and pathogens [88–90]. Some earlier investigations indicate that healthy *L. vannamei* and *M. rosenbergii* normally constitute THC levels of $103 \pm 4.6 \times 10^5$cells/mL [91]. In this study, THC of *L. vannamei* and *M. rosenbergii* were found within this range (Table 4),

clearly indicating good immunological or immunity status of the two species in the BFT system. For *L. vannamei*, significantly ($p < 0.05$) higher hemocyte counts were found in T_4 (15‰) compared to T_3 (10‰), T_2 (5‰), and T_1 (0‰), respectively (Table 4). According to [92–94], salinity is one of the important environmental stressors that can affect the physiology, immunology, growth, and survival of any aquatic species. Large-scale salinity change imposes osmotic stress on organisms that, in turn, reduce the THC of crustaceans and weakens the immunity system [95–98]. For *M. rosenbergii*, significantly ($p < 0.05$) higher hemocyte was found in T_1 (0‰) compared to T_2 (5‰), T_3 (10‰), and T_4 (15‰) (Table 4); these findings are similar to the earlier investigations [96, 99]. In-depth research is required to determine the change in THC is caused by cell proliferation, tissue-to-blood cell migration, or water osmosis between the medium and hemolymph for osmotic regulation [100].

4.4. Proximate Composition of PLs and Bioflocs. Salinity is known to play an important role in the biochemical makeup (moisture, lipid, and protein) of farmed crustaceans [82, 101, 102]. The findings of this study showed a similar trend with the earlier studies where protein, lipid, and ash contents of *L. vannamei* were significantly ($p < 0.05$) higher in T_4 (15‰) compared to T_3 (10‰), T_2 (5‰), and T_1 (0‰) (Table 5). Carbohydrate was highest at T_1 (0‰), with significant differences ($p < 0.05$) among the salinity treatments. According to Liang et al. [102], there was an inverse association between the amount of protein and carbohydrate content of crustaceans. For *L. vannamei*, crude protein, and lipid levels were considerably higher ($p < 0.05$) in T_4 (15‰) compared to T_3 (10‰), T_2 (5‰), and T_1 (0‰) (Table 5). An earlier investigation [98] found no significant variation in the body protein content of prawns within the salinity range of 0−20‰ but observed a positive relationship with increasing salinity, similar to the current research. For *M. rosenbergii*, ash and carbohydrate levels were considerably higher ($p < 0.05$) in T_1 (0‰) compared to T_2 (5‰), T_3 (10‰), and T_4 (15‰) (Table 5); these results are similar to the findings of [67]. Higher salinities can also negatively impact the growth of prawns due to osmotic stress that increases energy expenditure and lipid reserve depletion [44, 99]. In addition, the protein, lipid, and ash content of the bioflocs tended to increase with decreasing salinity (Table 6); showing similarity with the findings of [58, 103].

4.5. Growth Performance. L. vannamei exhibits good growth and production performance under a good farming environment with a wide range of salinities (5−40‰) [44]. In this study, the best survival rate for *L. vannamei* was in T_4 (15‰) (75.33 ± 5.03) and the lowest in T_1 (0‰) (24.00 ± 2.00) (Table 7). Some earlier studies obtained the best growth performance of *L. vannamei* at 10−15‰ salinity range, while the highest survival was found at 20‰ [28, 43, 83]. As *L. vannamei* is a marine species, lower salinities may have an adverse effect on physiology, which could lead to a decline in survival rate [104]. Survival rate (%) for *M. rosenbergii* was found to be significantly higher ($p < 0.05$) in T_2 (5‰) (93.33 ± 1.15) and T_3 (10‰) (92.00 ± 3.46) compared to T_4 (15‰) (88.67 ± 7.57) and T_1 (0‰) (87.33 ± 6.43) (Table 7). The best survival for *M. rosenbergii* was obtained at T_2 (5‰) (93.33) after 42 days of rearing, which was found to be similar with earlier investigations [105–107]. Adult *M. rosenbergii* can tolerate salinities ranging from 0 to 15‰, but the optimum range is ≈5‰ [99, 108]. Therefore, the highest survival rate of *M. rosenbergii* in T_2 (5‰) (Table 7) reflects the optimum salinity for this species, which is corroborating with earlier studies [109]. In the current study, FCR for *L. vannamei* was noticeably lower ($p < 0.05$) in T_4 (15‰) (1.21) than the other salinities which is similar to the findings of previous experiments [83, 101, 110]. In contrast, FCR for *M. rosenbergii* was observed to be noticeably lower ($p < 0.05$) in T_2 (5‰) (1.12) compared to the other salinities. In this experiment, lower SGR (%/day) for *L. vannamei* was recorded in T_1 (0‰) but *M. rosenbergii* exhibited significantly lower ($p < 0.05$) SGR in T_4 (15‰) (Table 7). Low salinity (≤5‰) negatively impacts the SGR of *L. vannamei* for nursery rearing in a biofloc system, while no such impact is observed for *M. rosenbergii* [58]. In this study, the growth performance of *L. vannamei* was negatively affected by the low-salinity, and *M. rosenbergii* was positively affected for nursery bi-culture in the biofloc system (Table 7). Earlier research [111] revealed that higher production of *L. vannamei* was obtained at high salinity conditions of 15–30‰. In a recent study, *M. rosenbergii* females raised at lower salinities of 0−6‰ produced more larvae than those raised at higher salinities of 12−18‰ [112]. According to [113–115], *M. rosenbergii* is an osmoregulator in freshwater; the iso-osmotic point is at medium salinity range (14−15‰), but is an osmoconformer at higher salinities (15−30‰). According to Tarlochan [116], freshwater prawns may grow in salinity as high as 17‰, with the greatest growth occurring in salinity between 0 and 5‰. Apart from the physiological stress, the growth of freshwater species can be impacted in higher salinities by increased energy consumption and protein sparing [45]. Energy in the form of protein [117–119] and/or lipids [120, 121] is known to be necessary for hyper-osmoregulation in aquatic crustaceans. Marine crustacean species grow less when exposed to low salinities due to decreased appetite and assimilation of food, respectively [120, 121].

5. Conclusion

The current study successfully investigated the nursery bi-culture potential of *L. vannamei* and *M. rosenbergii* in a biofloc system over a period of 42 days. Findings clearly indicate the highest level of production in T_4 (15‰). Typically, *L. vannamei* PL is grown at medium-to-high salinity environments, while *M. rosenbergii* PL is grown at freshwater conditions. Results obtained from this study clearly indicate that in the biofloc system, both species can be combined (bi-culture) in the T_4 (15‰), which will support better production and induction of aquaculture sustainability. It can be considered a climate-smart or climatically resilient technology. The findings of this study may improve the *L. vannamei* and *M. rosenbergii* in bi-culture by lowering feed costs and zero water exchange. It is important to note that successful

BFT requires optimization of salinity, species ratio, density, feeding rate, and frequency, as well as C:N ratio. This study successfully optimized the salinity requirement for these two commercially important species (bi-culture for nursery rearing) using BFT. The remaining other factors require optimization in the future to make this technology more profitable and sustainable.

Data Availability Statement

The information of this research is available from the corresponding author upon reasonable request.

Ethics Statement

The Institutional Animal Care and Use Committee (IACUC) at Universiti Putra Malaysia (UPM), Malaysia, approved the experimental methods involving animals (shrimp and prawn) in this study.

Conflicts of Interest

The authors declare no conflict of interest.

Author Contributions

Md. Abdul Halim, Aziz Arshad, Dania Aziz, and Nur Leena W.S. Wong contributed to conceptualization, methodology, investigation, data record, and formal analysis. Murni Karim, Md. Ariful Islam, Fadhil Syukri, and Md. Lifat Rahi contributed to data curation, checked journal format, and visualization. All authors have reviewed the published version of the manuscript and given their approval.

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