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Effects of C/N Ratio for Bi-Culture of *Litopenaeus vannamei* (Boone, 1931) and *Macrobrachium rosenbergii* (de Man, 1879) in a Molasses Based Biofloc System

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ABSTRACT

This study evaluated the effects of different C/N ratios (C10–N, C15–N, C20–N and C25–N, compared to C0–N as control) on growth, water quality, proximate composition, bacterial loads and total haemocyte counts (THCs) of shrimp (*Litopenaeus vannamei*) and prawn (*Macrobrachium rosenbergii*) post larvae in a biofloc-based nursery bi-culture system, aiming to optimize C/N ratios for sustainability and production efficiency over 6 weeks. Molasses was used as a carbon source, and water salinity was maintained at 15 ppt. In total, 15 cylindrical plastic tanks (125 L water-holding capacity, 59 cm × 56 cm) were used and filled up each 100 L water for rearing 1500 PL of the two species (750 *L. vannamei* and 750 *M. rosenbergii*). At the end of the experiment, significantly higher ($p < 0.05$) specific growth rate (SGR, % per day) (9.77) and survivability (95.33%) for *L. vannamei* were recorded in C10–N compared to the other treatments. For *M. rosenbergii*, the significantly higher ($p < 0.05$) SGR (% per day) (5.77) was recorded in C25–N, whereas the higher survival rate was found at C10–N (90.67%). The best feed conversion ratio was obtained at C10–N for *L. vannamei* (1.58), whereas for *M. rosenbergii*, it was obtained at C25–N (2.43). Significantly the higher ($p < 0.05$) THCs (*L. vannamei* $= 40.63 \times 10^5$ and *M. rosenbergii* $= 31.25 \times 10^5$) of both species were obtained at C10–N. The experimental C–N ratios led to a significant increase ($p < 0.05$) in total microbial loads ($116.6–355 \times 10^9$ CFU mL^{-1}) compared to the control (18.40×10^6 CFU mL^{-1}). Moreover, all tested water quality parameters remained within the optimal range across the treatments. The study concluded that a C:N ratio of 10:1 provides the highest economic return, making it the optimal ratio for biofloc-based nursery bi-culture of *L. vannamei* and *M. rosenbergii*.

1 | Introduction

Crustacean aquaculture is a key economic driver for many tropical and subtropical countries, especially in Asia and South America, producing 8.4 million tons valued at $9 billion in 2018 (Johnson, Masters, and Dalton 2022). Key crustacean species in global aquaculture include *Litopenaeus vannamei*, *Penaeus monodon*, *Macrobrachium nipponense*, *Macrobrachium rosenbergii* and *Scylla serrata* (Wang et al. 2022). *L. vannamei* accounts for 53% of global crustacean production, whereas *M. rosenbergii* contributes just 3% (El-Sayed 2017; FAO 2020). With its low global production, *M. rosenbergii* commands a market price for its gigantic size and delicious taste. *L. vannamei*, a South American native (marine) species, was introduced to Asian for its good aquaculture attributes, including salinity tolerance, faster growth, low feed conversion ratio (FCR), high survival rate, high density farming and polyculture potential (Davis, Samocha, and Boyd 2004; FAO 2009; Kumaran et al. 2017). *M. rosenbergii* is the most farmed freshwater crustacean significantly contributing to global freshwater aquaculture (Aziz et al. 2017). *M. rosenbergii* contributes significantly to aquaculture production and export earnings in countries like Bangladesh, India, Malaysia, Thailand and Vietnam (David et al. 2016; Pérez-Fuentes, Pérez-Rostro, and Hernández-Vergara 2013; Tidwell 2012). *M. rosenbergii* transitions from brackish waters for spawning to freshwater as it matures and is known for its strong environmental adaptability (Gao et al. 2022). To meet the demand of a growing global population, aquaculture is pursuing technological innovations to maximize food production from limited resources (FAO 2018). It will be high yielding, eco-friendly, sustainable, contamination-free and cost-effective (Burford et al. 2003).

Biofloc technology (BFT) is an emerging aquaculture technique that enhances production performance with minimal inputs, promotes biosecurity and supports wastewater recycling (Nisar et al. 2022). BFT is recognized as a microbial-based system that may boost the immune response of aquatic organisms (Kim et al. 2014). Biofloc use can improve survival and growth by enhancing digestive enzyme activity (Cardona et al. 2015; Long et al. 2015) and/or boosting immunity (Miao et al. 2017). BFT offers an economically viable, environmentally sustainable and socially acceptable approach to aquaculture (Ahmad et al. 2017; Crab et al. 2012). In aquaculture, the BFT can contribute to achieving sustainable development goals and making a cleaner environment (Bossier and Ekasari 2017). The composition, structure and stability of bioflocs are influenced by various organic carbon sources (such as molasses, corn, wheat, glucose, acetate, glycerol and tapioca) and their ratios (Panigrahi et al. 2018). Using molasses as a carbon source in BFT technology has been shown to positively impact shrimp growth, regulate inorganic nitrogen and phosphorus dynamics (Xu, Morris, and Samocha 2016) and enhance immunity (Ekasari et al. 2014). Different C–N ratios also impact the nutrient composition of bioflocs, including carbohydrate, protein, lipid and fatty acid content (Ekasari et al. 2014). For *L. vannamei*, C/N ratios of 10 and 15 (Chakrapani et al. 2021), 12 (Xu, Morris, and Samocha 2018) and 15–20 (Panigrahi et al. 2018) have been found to enhance growth performance, improve water quality and support immune function. In addition, several studies have shown that a C–N ratio of 20:1 leads to improved water quality in rearing tanks and better growth performance in *M. rosenbergii* (Hosain, Amin, Kamarudin, Arshad, Romano 2021c; Miao et al. 2017). No research has been done on the bi-culture of *L. vannamei* and *M. rosenbergii* in the BFT system.

BFT reduces space and water usage compared to semi-intensive systems, utilizing high stocking densities and minimal or no water exchange (Samocha et al. 2012). BFT is the leading technology driving the intensification of *L. vannamei* and *M. rosenbergii* production separately (Samocha et al. 2012). Biofloc-based nurseries reduce production costs, improve FCR, reduce nitrogen accumulation and enhance survival and juvenile health. (Antonio de Lorenzo et al. 2015; Correia et al. 2014; Emerenciano et al. 2011; Jatobá et al. 2014; Panjaitan, 2011; Panigrahi et al. 2018; Xu and Pan 2012). In the aquaculture phase, the co-culture of *L. vannamei* and *M. rosenbergii* in tidal ponds created a resource-efficient model that required no specialized feed, additional labour or extra capital (Ni et al. 2021). *L. vannamei* is a mid-layer inhabitant, whereas *M. rosenbergii* is a bottom inhabitant (Chong-Robles et al. 2014; David et al. 2016). 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.

Disease outbreaks have inflicted significant economic losses (*L. vannamei*), with estimates reaching approximately $4 billion annually between 2009 and 2018 (Shinn et al. 2018). Early stages of *L. vannamei* and *M. rosenbergii* are highly vulnerable to pathogens leading to significant mortality and financial loss in the industry (Panigrahi et al. 2020; Rajendran et al. 2016). The nursery phase (post larvae to juvenile) is crucial for uniform growth, better feed utilization and higher survival in *L. vannamei* and *M. rosenbergii* farming (Fóes et al. 2011). Earlier research has shown that successful nursery rearing leads to higher yields and profits in grow-out farming (Coyle, Alston, and Sampaio 2010; Fóes et al. 2011; Hosain, Amin, Arshad et al. 2021; Hosain, Amin, Kamarudin, Arshad, Karim et al. 2021; Hosain, Amin, Kamarudin, Arshad, Romano 2021). No studies have yet focused on optimizing the C–N ratio of *L. vannamei* and *M. rosenbergii* in a biofloc system.

Species selection is key to BFT success. Target species must have a high market value, adapt to commercial and live feed, grow at high stocking densities and utilize different water column positions. Both *L. vannamei* and *M. rosenbergii* possess these traits, as shown in previous studies (Hoang, Nguyen, and Bossier 2020; Martínez-Porchas et al. 2010; Yuan et al. 2010). The production performance of *L. vannamei* and *M. rosenbergii* can be evaluated by culturing them together in a nursery-based biofloc system due to their high economic value in aquaculture. In order to do this, trials must optimize different parameters, including stocking density, salinity level, C–N ratio, feeding frequency and rates. The main objective of this study is to determine the optimal C–N ratio for bi-culture of *L. vannamei* and *M. rosenbergii* in the nursery phase. By establishing the ideal C–N ratio, this research will contribute to sustainable practices in aquaculture with innovative potential of bi-culture for these commercially valuable species.

2 | Materials and Methods

2.1 | Experimental Procedure (Acclimation Periods) and Species Collection

The current study was conducted over a 6 weeks' time frame (from August to September 2023) at the International Institute of Aquaculture and Aquatic Sciences (I-AQUAS), University Putra Malaysia (UPM). Prior to the start of this experiment, two round fibre tanks (1000 L) were prepared and filled with 15 ppt water. Both tanks were maintained with continuous and vigorous aeration (three air stones inserted at the three sides) for 3 days. Then 1500 *L. vannamei* and 1500 *M. rosenbergii* PL_{10} were collected from GIMACRO ENTERPRISE, KG Jelutong, 32020 Sitiawan Peraka, Malaysia. *L. vannamei* and *M. rosenbergii* were maintained in separate tanks (1000 L round fibre tanks) for acclimation with the laboratory condition (in round fibre tanks) for 4 days. A commercial shrimp and prawn meal pellet feed (40% crude protein; minuscule crumbs, 0.25–1 mm in size) was provided at the rate of 75% of body weight to the PLs three times daily (at 9:00 AM, 2:00 PM and 6:00 PM) for acclimation.

2.2 | Starter Preparation

Two probiotic strains (*Lysinibacillus fusiformis* SPS11, *Enterococcus hirae* LAB3) were used to prepare the starter for this experiment. The probiotic strains were collected from the Laboratory of Fish Health, Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia (UPM). The concentration of the probiotic strains was 5×10^8 CFU mL^{-1}. Biofloc starter was prepared according to the methods outlined in Hosain, Amin, Arshad et al. (2021), Hosain, Amin, Kamarudin, Arshad, Karim et al. (2021), Negrini et al. (2017), Pekkoh et al. (2022), Zabidi et al. (2021) and Wang et al. (2019). To prepare the biofloc starter, 55 mL (0.055 L) of molasses (carbohydrate source) and 1 L of probiotics consortium (*L. fusiformis* SPS11 and *E. hirae* LAB3) (5×10^8 CFU mL^{-1}) were mixed with 5.50 L of saline water (15‰) in a bucket and mixed well to dissolve the ingredients thoroughly to prepare 6.555 L of starter according to the protocols mentioned above. The bucket containing the entire mixture was sealed with the aluminium foil to keep it free from contamination and maintained at ambient temperature for 6 days to complete an aerobic fermentation (a sweet smell indicated the growth of target probiotic bacteria). This fermented starter was used as the stock or mother solution to apply in the experimental tanks. Following the starter preparation, the entire mixture (6.555 L) was divided in 12 equal parts to apply in 12 treatment tanks (546.25 mL $tank^{-1}$). No starter was added in the three control tanks.

2.3 | Experimental Design and Procedure (Tanks Setting)

A total of 15 cylindrical plastic tanks (100 L each) were used to investigate the effects of 5 different carbon–nitrogen (C–N) ratios (3 replicated tanks for each C–N ratio) on the production performance of *L. vannamei* and *M. rosenbergii* in a nursery-based bi-culture (Table 1). The experimental tanks were filled with 15‰ water prior to the start of this experiment and maintained with continuous aeration. Following this step, 546.25 mL starter was

TABLE 1 | Description of different C–N ratio-based biofloc systems used for the experiment of *Litopeneus vannamei* and *Macrobrachium rosenbergii* post larvae.

Group	Descriptions	Stocking ration	Number of shrimp/prawn PL (PLs × replicate)
C–N0	Clean water with 15 ppt (30% exchange daily)	50V:50M	(V = 50 + M = 50) × 3 = 300
C–N10	C–N10 treatment was carbon/nitrogen ratio 10:1 with 15 ppt salinity-based biofloc system		(V = 50 + M = 50) × 3 = 300
C–N15	C–N15 treatment was carbon/nitrogen ratio 15:1 with 15 ppt salinity-based biofloc system		(V = 50 + M = 50) × 3 = 300
C–N20	C–N20 treatment was carbon/nitrogen ratio 20:1 with 15 ppt salinity-based biofloc system		(V = 50 + M = 50) × 3 = 300
C–N25	C–N25 treatment was carbon/nitrogen ratio 25:1 with 15 ppt salinity-based biofloc system		(V = 50 + M = 50) × 3 = 300

Note: V = *Litopenaeus vannamei*, M = *Macrobrachium rosenbergii*.

added in each of the experimental tanks. Floc concentrations were measured after 3 days for each tank and were found as 0.5–1.00 mL L^{-1} (Avnimelech 1999), which is suitable for stocking *L. vannamei* and *M. rosenbergii* PL. In each tank, 100 PL were stocked at an equal ratio (50 *L. vannamei* + 50 *M, rosenbergii*): 300 individuals per experimental condition (Indulkar and Belsare 2004). At the start of this experiment, the initial body weight of *L. vannamei* was 0.0286 ± 0.0004 g and for *M. rosenbergii*, it was 0.0253 ± 0.0001 g.

2.4 | Feeding the Experimental Animals

A commercial shrimp feed manufactured by CRP Dindings (40% crude protein, 5% fat, 4% crude fibre and 12% moisture) was applied for the experimental *L. vannamei* and *M. rosenbergii* PLs four times daily (8:00 AM, 12:00 PM, 3:00 PM and 6:00 PM). Initially, feed was applied at the rate of 20% of total biomass, which was gradually adjusted to 10% according to the methods outlined in de Marques, Lombardi, and Boock (2000), Emerenciano et al. (2011). The digestibility of the feed (CRP Dindings) is 30%, and it contents at 90% of dry matter.

2.5 | Carbon Source and Experimental Doses

Molasses was added in the experimental tanks as a carbon source, which was added at different doses to maintain four different C–N ratios: 10:1, 15:1, 20:1 and 25:1. To achieve these C–N ratios, molasses was added according to the equation of Wei, Liao, and Wang (2016). In addition, molasses was added daily in the experimental tanks to maintain the target C–N ratios using the same equations. Abakari, Luo, and Kombat (2021) and De Schryver et al. (2008) stated that normally every feed contains 50% carbon when it is formulated. The daily amount of carbon: nitrogen (C:N ratio) was calculated according to the feed supply's overall protein level. By multiplying the quantity of carbon by 0.31 (molasses contains 31.88% organic carbon, according to testing by the Material Characterization Laboratory, UPM). Molasses was added daily in the experimental tanks in the morning (11:00 AM) according to Table 2.

2.6 | Water Quality Parameters

Water pH and temperature were measured daily at 9:00 AM, using a YIERYI Digital pH Meter (YIERYI 3-in-1 PH Tester). Ammonia (NH_3/NH_4^+), nitrate (NO_3^-) and nitrite (NO_2^-) were measured every day at 10:00 AM using API test kit (MARS Fishcare North America). Dissolved oxygen (DO) (ppm) was measured every week at 8:30 AM, using a Doc SMART SENSOR Digital DO Meter. Total dissolved solids (TDS) (ppm) were measured every week at 9:30 AM, using a TDS EC (3 in 1 LCD digital) water purity tester. Bioflocs volume (BFV) was measured with the aid of Imhoff cones (after 30 min sedimentation of 1 L of water samples) every day according to Avnimelech (2009).

TABLE 2 | Daily doses of molasses to maintain the target C–N ratios in experimental tanks.

C–N ratio									
C–N = 0 (control)		**C–N = 10:1**		**C–N = 15:1**		**C–N = 20:1**		**C–N = 25:1**	
0 mL	T_1R_1 (0)	1.10 mL	T_2R_1 (0.37)	3.50 mL	T_3R_1 (1.17)	6.00 mL	T_4R_1 (2.00)	8.1 mL	T_5R_1 (2.70)
	T_1R_2 (0)		T_2R_2 (0.35)		T_3R_2 (1.15)		T_4R_2 (2.10)		T_5R_2 (2.60)
	T_1R_3 (0)		T_2R_3 (0.38)		T_3R_3 (1.18)		T_4R_3 (1.90)		T_5R_3 (2.80)

2.7 | Microorganisms

At the end of this experiment, total beneficial/probiotic bacterial (*L. fusiformis* SPS11 and *E. hirae* LAB3) loads were calculated. Tryptic soya agar media (TSA; Difco, Detroit, MI, USA) was used for the estimation of total bacterial loads with the dilution factors of 10^6, 10^7 and 10^8. For subsequent investigation, a well-mixed 10 mL sample from each tank was collected (Emerenciano, Gaxiola, and Cuzo 2013; Thompson, Abreu, and Wasielesky 2002). Total bacterial loads were determined by the following equation:

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

2.8 | Total Haemocyte Counts (THCs) of the Two Species

A total of 5 *L. vannamei* and 5 *M. rosenbergii* were randomly sampled from each tank (a total of 150 individuals: 30 from each experimental condition). Samples were then disinfected by soaking in 70% ethanol to prevent contamination. From each species, 50 μL of haemolymph sample was collected by micro-injection inserted through the ventral sinus of the first abdominal segment (Chen et al. 2015). Each syringe was pre-filled with 100 μL of anti-coagulant 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^{-1} by the addition of 115 mM glucose] to prevent clotting of haemolymph samples (Chen et al. 2015). The anticoagulated haemolymph sample was transferred into a Neubauer Haemocytometer (Loptik Labour, Germany) for inspection under a light microscope (BX43, OLYMPUS) to count the number of total haemocytes. The Neubauer chamber has a volume of 0.0025 mm^3 and a depth of 0.1 mm. To calculate the average value, the THC from two Neubauer chamber partitions was divided into two equal parts and referred to as A and B, respectively (Chuan and Din 2010). Five compartments were counted from every segment to represent the entire part. THCs were finally counted by the following formula:

$$\text{THC (Cell mL} - 1) = \frac{\text{Number of cells} \times 10000}{\text{Number of square} \times \text{dilition factor}}.$$

2.9 | Proximate Analysis

Biofloc samples were collected from each tank by using a plankton net (mesh size 60 μm), whereas shrimp and prawn samples were collected by scope net at the end of this experiment. The biofloc, shrimp and prawn samples were dried in an oven at 55°C until they reached a constant weight, and they were kept at −20°C for subsequent analysis. Using AOAC standard procedures, the proximate compositions of shrimp, prawn and biofloc were examined in triplicate (AOAC 1990). The amount of ash was determined by burning the samples at 600°C for 5 h. Lipid contents of the samples were ascertained in a lipid analyser (Foss Tecator, Soxtec8000). The amount of protein was quantified using a protein analyser following a 60 min of acid digestion step. The amount of total carbohydrate was calculated according to the following formula (Manush et al. 2005):

$$\text{Carbohydrate (\%DW)} = 100 - [\text{crudeprotein (\%DW)} + \text{lipid (\%DW)} + \text{ash (\%DW)}]$$

TABLE 3 | Different aspects of water quality parameters in the bi-culture-based biofloc systems for rearing *Litopeneus vannamei* and *Macrobrachium rosenbergii* post larvae for 42 days.

Parameters	C–N ratio					*p* value
	C–N = 0 (control)	C–N = 10:1	C–N = 15:1	C–N = 20:1	C–N = 25:1	
pH (ppm)	7.51 ± 0.01^a	7.25 ± 0.06^b	7.26 ± 0.04^{bc}	7.25 ± 0.01^b	7.27 ± 0.03^c	0.001
Ammonia (ppm)	(0–1.58)^a	(0–0.37)^b	(0–0.38)^b	(0–0.41)^c	(0–0.38)^b	0.001
Temperature (°C)	27.96 ± 0.01^a	27.92 ± 0.04^b	27.99 ± 0.03^c	28.02 ± 0.02^d	27.99 ± 0.04^c	0.001
DO (ppm)	5.67 ± 0.10^a	5.54 ± 0.07^b	5.48 ± 0.10^c	5.53 ± 0.14^b	5.53 ± 0.17^b	0.001
Nitrate (ppm)	(0–32.14)^a	(0–14.40)^b	(0–14.53)^c	(0–15.48)^d	(0–21.90)^e	0.001
Nitrite (ppm)	(0–0.74)^a	(0–0.39)^b	(0–0.51)^c	(0–0.43)^d	(0–0.74)^a	0.001
TDS (ppm)	8525.71 ± 90.32^a	8901.43 ± 22.27^a	8678.57 ± 35.72^a	8610.33 ± 52.30^a	8607.14 ± 14.29^a	0.555
Floc volume (mL L^{-1})	—	10.48 ± 1.91^d	12.62 ± 1.08^c	13.26 ± 0.23^b	14.83 ± 1.85^a	0.001

Note: Mean ± SD values in same row with a similar superscript are not noticeable different ($p > 0.05$), and different letters differed ($p < 0.05$) significantly.
Abbreviation: TDS, total dissolved solids.

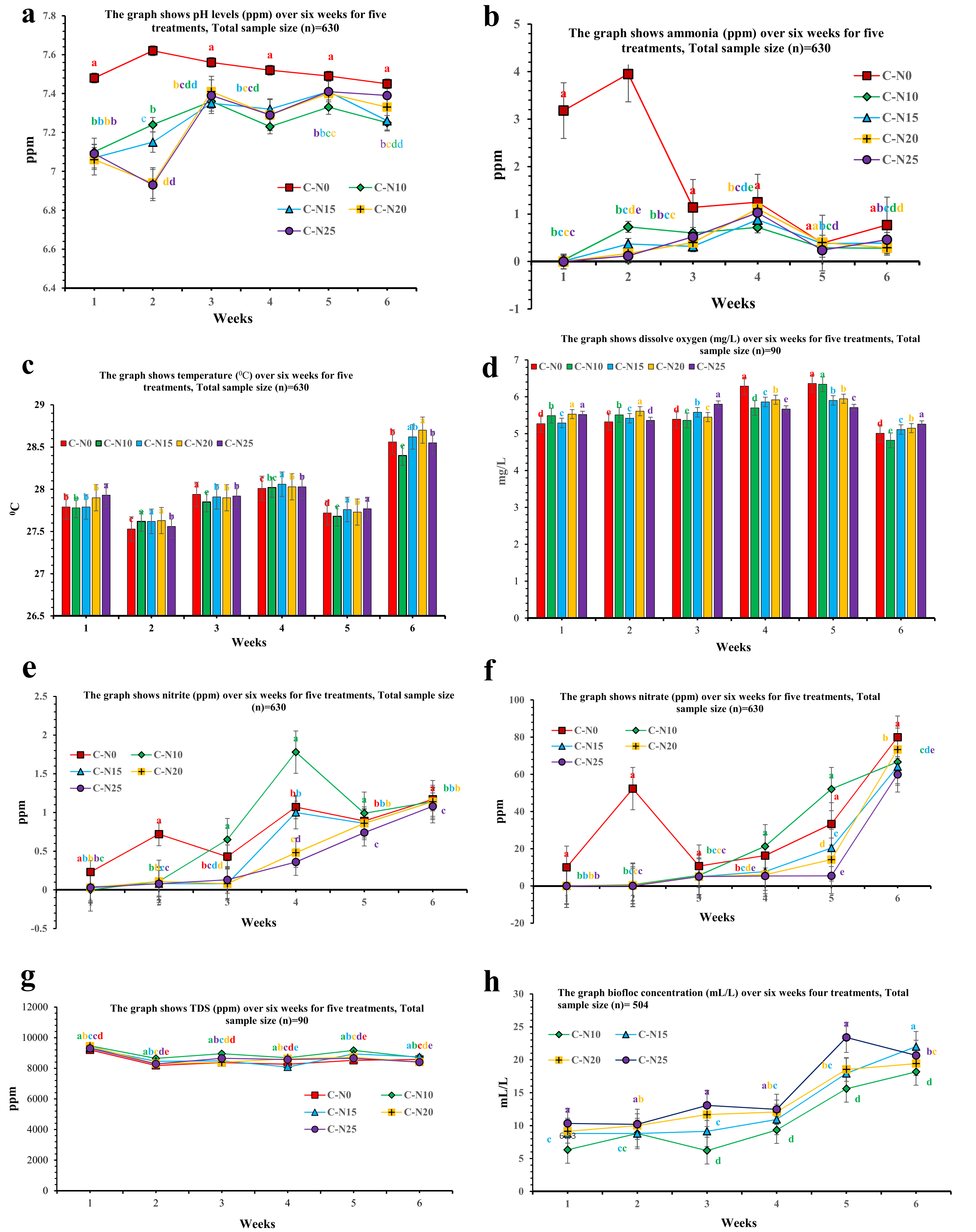

FIGURE 1 | Weekly mean (±SD) pH (a), ammonia (NH_3/NH_4^+) (b), temperature (°C) (c), dissolve oxygen (DO) (d), nitrite (ppm) (e), nitrate (ppm) (f), total dissolved solids (TDS) (ppm) (g) and biofloc concentration (mL L^{-1}) (h) in BFT applying five distinct treatments in culturing *Litopenaeus vannamei* and *Macrobrachium rosenbergii* post larvae for 42 days. Different letters indicate significant difference ($p < 0.05$) as per Duncan's multiple range tests. BFT, Biofloc technology.

TABLE 4 | Total bacterial loads (CFU mL^{-1}) (after 42 days of rearing) in five different treatments using biofloc systems for rearing *Litopenaeus vannamei* and *Macrobrachium rosenbergii* post larvae.

C–N ratio					
C–N0 (control)	**C–N10**	**C–N15**	**C–N20**	**C–N25**	***p* value**
18.40×10^{6a}	116.60×10^{9b}	179.20×10^{9c}	193.70×10^{9d}	355.00×10^{9e}	0.001

Note: Values in same row with a similar superscript are not noticeable different ($p > 0.05$), and different letters differed ($p < 0.05$) significantly.

TABLE 5 | Total haemocyte counts (cells mL^{-1}) of *Litopenaeus vannamei* and *Macrobrachium rosenbergii* post larvae 42 days post rearing in a biofloc system to test the bi-culture potential of the two species.

Species	**C–N ratio**					
	Concentration (number of cells mL^{-1}) [10^5]					
	C–N0 (control)	**C–N10**	**C–N15**	**C–N20**	**C–N25**	***p* value**
L. vannamei	21.63^a	40.63^b	33.25^c	29.75^d	24.43^e	0.001
M. rosenbergii	18.75^a	31.25^b	30.63^c	23.5^d	18.75^a	0.001

Note: Values in same row with a similar superscript are not noticeable different ($p < 0.05$), and different letters differed ($p < 0.05$) significantly.

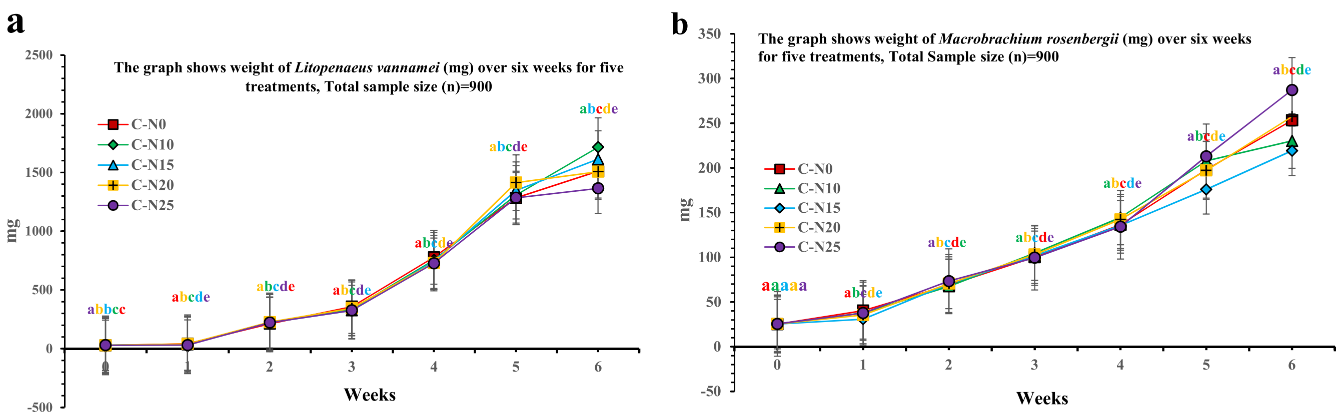

FIGURE 2 | Weekly mean (±SD) weight of *Litopenaeus vannamei* (a) and *Macrobrachium rosenbergii* (b) post larvae reared biofloc systems using five different treatments during a 42-day culture period. Different letters indicate significant difference ($p < 0.05$) as per Duncan's multiple range tests.

2.10 | Growth Parameter and Economic Analysis

From each tank, 5 shrimp (*L. vannamei*) and 5 prawn (*M. rosenbergii*) samples were randomly collected (30 individuals per treatment) once a week to measure growth performance. The survival rates of prawn and shrimp samples were determined by counting the total number of survivors at the end of this study (after 42 days of rearing in the biofloc system). The final weight, weight gain, FCR, specific growth rate (SGR) and survival rate were calculated using the following formulae (Maicá et al. 2012):

$$\text{SGR}\ (\%\ \text{day} - 1) = \frac{\ln W_f - \ln W_i}{t} \times 100$$

where W_f = final weight, W_i = initial weight and t = time in days

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

$$\text{Daily weight gain (DWG) (gm day} - 1) = \frac{W_t - W_0}{t}$$

where $W_{t.}$ and W_0 are the final and initial body weight of the shrimp/Prawn, respectively, and t is the total duration of the grow-out trial in days.

$$\text{Biomassgain (g)} = \text{Harvestedbiomass (g)} - \text{stockedbiomass (g)}$$

$$\text{Feed conversion ratio} = \frac{\text{Total amount feed given (g)}}{\text{Weight gain (g)}}$$

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

TABLE 6 | Proximate composition of *Litopenaeus vannamei* and *Macrobrachium rosenbergii* post larvae after 42 days of rearing in a biofloc system.

Species	Variable	C–N ratio					*p* value
		C–N0 (Control)	C–N10	C–N15	C–N20	C–N25	
L. vannamei	Protein	59.10 ± 0.90^{a}	60.50 ± 0.75^{b}	61.80 ± 0.60^{c}	64.60 ± 0.70^{d}	65.10 ± 0.85^{e}	0.001
	Lipid	8.10 ± 0.25^{a}	9.70 ± 0.30^{b}	10.50 ± 0.20^{ab}	10.70 ± 0.40^{b}	11.00 ± 0.85^{b}	0.001
	Ash	13.40 ± 0.90^{a}	13.90 ± 0.50^{b}	14.80 ± 0.15^{c}	15.50 ± 0.76^{d}	15.70 ± 0.83^{e}	0.001
	Carbohydrate	19.40 ± 0.85^{a}	15.90 ± 0.50^{b}	12.90 ± 0.46^{c}	9.20 ± 0.55^{d}	8.20 ± 0.35^{e}	0.001
M. rosenbergii	Protein	62.10 ± 0.55^{a}	63.50 ± 0.20^{b}	63.70 ± 0.80^{bc}	64.40 ± 0.25^{c}	65.70 ± 0.55^{d}	0.001
	Lipid	3.00 ± 0.09^{a}	4.10 ± 0.25^{b}	5.90 ± 0.40^{c}	7.60 ± 0.50^{d}	8.30 ± 0.44^{e}	0.001
	Ash	14.30 ± 0.65^{a}	14.80 ± 0.35^{b}	15.50 ± 0.50^{c}	15.90 ± 0.55^{d}	16.10 ± 0.60^{e}	0.001
	Carbohydrate	20.60 ± 0.25^{a}	17.60 ± 0.30^{b}	14.90 ± 0.56^{c}	12.10 ± 0.70^{d}	9.90 ± 0.80^{e}	0.001

Note: Mean ± SD values in same row with a similar superscript are not noticeable different ($p < 0.05$), and different letters differed ($p < 0.05$) significantly.

2.11 | Economic Analysis

A basic algebraic economic analysis was conducted to determine the net profit and benefit–cost ratio for the culture of *L. vannamei* and *M. rosenbergii* under various treatments. The following equation was used to calculate the profitability of *L. vannamei* and *M. rosenbergii* cultured in BFT systems:

$$NP = GR - TC$$

where NP is the net profit, GR is the gross return from the sales of *L. vannamei* and *M. rosenbergii*, and TC is the total cost (fingerlings, feed and others).

This formula was used to calculate the benefit–cost ratio:

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

2.12 | Statistical Analysis

The SPSS (version 25) program was used to conduct the statistical tests (particularly one-way analysis of variance [ANOVA]. The Shapiro–Wilks and Levene's tests were used to determine the uniformity and normality of the variances. The differences between treatments were examined using an ANOVA. The post hoc assessment of the mean across distinct groups was done using Duncan's multiple range tests, whereas significant variations were found at the 5% level of significance ($p < 0.05$). In the tables, all of the data were displayed as mean ± SD, and significant differences were at $\alpha = 5\%$.

3 | Results

3.1 | Water Quality Parameters

Table 3 and Figure 1 display different types of water quality parameters measured in this study. Significantly higher ($p < 0.05$) pH (ppm), ammonia (NH_3/NH_4^+) (ppm), temperature (°C), DO (ppm), nitrate (NO_3^-) (ppm), nitrite (NO_2^-) (ppm) and floc volume (mL L^{-1}) were observed for the control group over the treatments. There were no significant differences ($p > 0.05$) in TDS (ppm) levels among the treatments.

3.2 | Total Microbial (Bacterial) Loads

Experimental carbon–nitrogen (C–N) ratios significantly increased ($p < 0.05$) the total microbial loads (116.60–355 × 10^9 CFU mL^{-1}) in treatment groups compared to the control (18.40 × 10^6 CFU mL^{-1}) (Table 4). Total bacterial loads were observed to be in an increasing trend with increasing C:N ratios, which were ranked as C–N0 (control = (18.40 × 10^6) < C–N10 (116.60 × 10^9) < C–N15 (179.20 × 10^9) < C–N20 (193.70 × 10^9) < C–N25 (355 × 10^9) (Table 4).

TABLE 7 | Proximate composition of biofloc in a bi-culture system of *Litopenaeus vannamei* and *Macrobrachium rosenbergii* post larvae after 42 days of rearing.

Variable	C–N ratio					*p* value
	C–N0	C–N10	C–N15	C–N20	C–N25	
Protein	—	24.40 ± 0.40^{a}	24.60 ± 0.90^{a}	26.20 ± 0.85^{b}	26.90 ± 0.70^{b}	0.001
Lipid	—	0.10 ± 0.02^{a}	0.90 ± 0.03^{b}	1.80 ± 0.09^{c}	2.10 ± 0.25^{d}	0.001
Ash	—	23.50 ± 0.56^{a}	25.70 ± 0.40^{b}	26.60 ± 0.75^{c}	26.70 ± 0.80^{c}	0.001
Carbohydrate	—	52.00 ± 0.95^{a}	48.80 ± 0.85^{b}	45.40 ± 0.75^{c}	44.30 ± 0.85^{d}	0.001

Note: Mean ± SD values in same row with a similar superscript are not noticeable different ($p < 0.05$), and different letters differed ($p < 0.05$) significantly.

3.3 | THCs of the Two Species

Experimental C–N ratios significantly altered ($p < 0.05$) the THCs of *L. vannamei* and *M. rosenbergii* (Table 5). The highest numbers of haemocyte counts were observed at C–N10 (40.63×10^5 for *L. vannamei* and 31.25×10^5 for *M. rosenbergii*), whereas the lowest numbers were observed at control (CN0 = 21.63×10^5 and 18.75×10^5) for both species (Table 5).

3.4 | Proximate Composition of *L. vannamei* and *M. rosenbergii*

Table 6 shows that experimental C–N ratios significantly ($p < 0.05$) affected the proximate composition of *L. vannamei* and *M. rosenbergii*. Protein, lipid and ash contents were found to increase significantly ($p < 0.05$) with raising C–N ratios for both species. All four treatment groups (C10–N, C15–N, C20–N and C25–N) exhibited significantly higher protein, lipid and ash contents over the control (C0–N). Carbohydrate content was found in a declining trend with increasing C–N ratios.

3.5 | Proximate Composition of Biofloc

Proximate composition of the biofloc showed a similar trend as observed for shrimp and prawn: A significant increase ($p < 0.05$) in protein, lipid and ash contents with increasing C–N ratios, whereas there is a significantly declining ($p < 0.05$) trend for carbohydrate content (Table 7). Significant differences were observed among the four treatments for lipid and carbohydrate contents. Significantly lower protein content ($p < 0.05$) was observed for C10–N and C15–N (no significant difference between these two) compared to C20–N and C25–N (no significant difference among them). A similar pattern was observed for ash content (Table 7).

3.6 | Growth Parameters

Growth performance and economic analysis of *L. vannamei* and *M. rosenbergii* raised in five different experimental conditions have been presented in Tables 8 and 9 and Figure 2. A general declining trend in growth (final body weight) was observed with increasing C–N ratios; the highest level of growth was obtained at C10–N. Growth of *L. vannamei* among the experimental conditions was ranked as C25–N = 1.36 g < C20–N = 1.51 g < control (C0–N = 1.51 g) < C15–N = 1.61 g < C10–N = 1.71 g. The highest level of survival rate for *L. vannamei* was observed at C10–N (95.33 ± 1.15) and the lowest at C0–N (Control = 82.67 ± 8.08). The same pattern was observed for *M. rosenbergii*: The highest survival performance at C10–N (90.67 ± 2.31) and the lowest at C0–N (Control = 73.33 ± 5.77). Similar to growth and survival performance, other production-related parameters (including SGR, weight gain, biomass gain, species-specific production and total production) were found to be higher at C10–N with relatively low FCR for both species. Total productions (g) of the two species were found in the rank of C0–N (control = 71.72 g) < C25–N (72.22 g) < C20–N (78.87 g) < C15–N (83.71 g) < C10–N (92.16 g). Results clearly indicate that the optimum C–N ratio is 10:1 for maximum production of *L. vannamei* and *M. rosenbergii* in a biofloc-based nursery bi-culture system.

3.7 | Economic Analysis

The economic analysis clearly revealed the suitability of C–N ratio 10:1 for maximum profitability (Table 9) in biofloc-based nursery bi-culture of *L. vannamei* and *M. rosenbergii*. Statistical analysis indicates significantly higher ($p < 0.05$) gross return, net profit and BCR at C10–N while significantly lower investment.

4 | Discussion

BFT is known to improve water quality by reducing nitrogenous waste (Long et al. 2015), and minimize excessive water use and environmental concerns through reduced effluent discharge (Azim and Little 2008; Nisar et al. 2022). The efficiency of BFT depends on optimizing different parameters for target species, of which carbon–nitrogen (C–N) ratio is very crucial. Earlier investigations indicate that the optimum range of different water quality parameters for shrimp (*L. vannamei*) or prawn (*M. rosenbergii*) include: DO = 5–8 ppm, pH = 6.80–8.20, ammonia = 0.06–0.20 ppm, temperature = 26°C–30°C, nitrate = 0.30–1.20 ppm, nitrite = 0.02–0.10 ppm and floc concentration = 6–80 mL L^{-1} (Ballester et al. 2017; Correia et al. 2014; Gaona et al. 2017; Hosain, Amin, Arshad et al. 2021; Hosain, Amin, Kamarudin, Arshad, Karim et al. 2021; Hosain, Amin, Kamarudin, Arshad, Romano 2021; Negrini et al. 2017; Peixoto et al. 2018; Pérez-Fuentes, Pérez-Rostro, and Hernández-Vergara 2013; Rajkumar et al. 2016; Schveitzer et al. 2013; Xu and Pan 2012, 2014). In addition to these parameters, the amount of TDS is an important factor that is suitable in a range of 16,590–19,760 mg L^{-1} for brackish water biofloc systems (Elhetawy et al. 2021). In this

TABLE 8 | Survival rate (%), FCR, SGR (% day^{-1}), weight gain (%), daily weight gain (g day^{-1}), biomass gain (g) and production (g) of *Litopenaeus vannamei* and *Macrobrachium rosenbergii* post larvae after 42 days of rearing in biofloc system.

Parameters	Species	C–N ratio					p value
		C–N0 (control)	C–N10	C–N15	C–N20	C–N25	
Initial weight (g)	V	0.0281 ± 0.00^{a}	0.0283 ± 0.10^{a}	0.0287 ± 0.00^{b}	0.0288 ± 0.00^{bc}	0.0290 ± 0.00^{c}	0.001
	M	0.0252 ± 0.00^{a}	0.0252 ± 0.00^{a}	0.0253 ± 0.00^{a}	0.0254 ± 0.00^{a}	0.0255 ± 0.00^{a}	0.442
Final weight (g)	V	1.51 ± 0.26^{a}	1.72 ± 0.19^{b}	1.61 ± 0.20^{c}	1.51 ± 0.27^{a}	1.36 ± 0.20^{d}	0.001
	M	0.25 ± 0.00^{a}	0.23 ± 0.00^{b}	0.20 ± 00^{c}	0.26 ± 00^{d}	0.29 ± 00^{e}	0.001
Survival rate (%)	V	82.67 ± 8.08^{a}	95.33 ± 1.15^{b}	92.67 ± 3.06^{b}	90.00 ± 8.72^{c}	89.33 ± 8.33^{c}	0.001
	M	73.33 ± 5.77^{a}	90.67 ± 2.31^{b}	82.00±14.00^{c}	86.00 ± 5.29^{d}	78.67 ± 8.08^{e}	0.001
FCR	V	2.07^{a}	1.58^{b}	1.72^{b}	1.95^{a}	2.05^{a}	0.001
	M	2.89^{a}	2.83^{a}	3.02^{b}	2.44^{c}	2.43^{c}	
SGR (% day^{-1})	V	9.48^{a}	9.77^{b}	9.60^{c}	9.42^{d}	9.17^{e}	0.001
	M	5.50^{a}	5.24^{b}	5.14^{c}	5.51^{a}	5.77^{d}	0.001
Weight gain (%)	V	5271.27^{a}	5951.89^{b}	5528.62^{c}	5127.97^{d}	4601.24^{e}	0.001
	M	905.44^{a}	813.42^{b}	766.75^{c}	912.07^{d}	1026.72^{e}	0.001
Daily weight gain (g day^{-1})	V	0.035^{a}	0.040^{b}	0.038^{c}	0.035^{a}	0.032^{d}	0.001
	M	0.005^{a}	0.005^{a}	0.005^{a}	0.006^{b}	0.006^{b}	0.001
Biomass gain (g)	V	1.48^{a}	1.69^{b}	1.58^{c}	1.48^{a}	1.34^{d}	0.001
	M	0.23^{a}	0.21^{b}	0.19^{c}	0.23^{a}	0.26^{d}	0.001
Production (g)	V	62.43^{a}	81.72^{b}	74.72^{c}	67.80^{d}	60.94^{e}	0.001
	M	9.29^{a}	10.44^{b}	8.99^{c}	11.07^{d}	11.28^{e}	0.001
Total production (g)		71.72^{a}	92.16^{b}	83.71^{c}	78.87^{d}	72.22^{a}	0.001

Note: V = *Litopenaeus vannamei*, M = *Macrobrachium rosenbergii*. Mean ± SD values in same row with a similar superscript are not noticeable different ($p < 0.05$), and different letters differed ($p < 0.05$) significantly.
Abbreviations: FCR, feed conversion ratio; SGR, specific growth rate.

TABLE 9 | Economic analysis for the bi-culture of *Litopenaeus vannamei* and *Macrobrachium rosenbergii* post larvae after 42 days of rearing in biofloc system.

Parameters	C–N ratio					*P* value
	C–N0 (control)	C–N10	C–N15	C–N20	C–N25	
Total cost (USD)	6.12^{a}	6.06^{b}	6.08^{c}	6.10^{d}	6.09^{c}	0.001
Gross return (USD)	15.19^{a}	17.97^{b}	17.14^{c}	17.19^{d}	16.36^{e}	0.001
Net profit (USD)	9.08^{a}	11.90^{b}	11.06^{c}	11.09^{c}	10.26^{d}	0.001
BCR	1.48^{a}	1.96^{b}	1.82^{c}	1.82^{c}	1.68^{d}	0.001

Note: Values in same row with a similar superscript are not noticeable different ($p < 0.05$), and different letters differed ($p < 0.05$) significantly. PL rate 0.029 USD, feed 0.924 USD kg^{-1} and selling price 0.242 USD/*Litopenaeus vannamei* and 0.147 USD/*Macrobrachium rosenbergii*. 1RM = 0.21 USD. This financial evaluation did not take into account the lab equipment that provided I-AQUAS or the Department of Aquaculture, Universiti Putra Malaysia.
Abbreviation: BCR, benefit–cost ratio.

study, all of these water quality parameters (DO, temperature, pH, ammonia, nitrate, nitrite, floc concentration and TDS) were found to be within the optimum range (Table 3) for the treatment groups (C–N ratios = 10:1, 15:1, 20:1 and 25:1); clearly indicating the important functions of different C–N ratios in the biofloc systems for improving water quality compared to the control (C0–N). The findings of this study also imply that the best performance (in terms of different water quality parameters) for the nursery bi-culture of Pacific white shrimp (*L. vannamei*) and giant prawn (*M. rosenbergii*) in biofloc system was obtained at C–N = 10:1.

Heterotrophic bacteria in the biofloc system produce microbial/bacterial proteins (highly nutritious single-cell proteins), providing high quality feed supplement (also reducing the demand/amount of artificial/supplementary feed), enhancing growth and immunity, ensuring proper utilization of feed for the target species (Ebeling, Timmons, and Bisogni 2006; Schveitzer et al. 2013). In addition, the beneficial microbial community in BFT is capable of absorbing feed wastes (detoxification and purification of water), converting the nitrogenous metabolites (e.g., ammonia) of wastes into microbial proteins and, therefore, reducing loads of harmful microbes (Browdy et al. 2001; Correia et al. 2014; Kim et al. 2014; Panigrahi et al. 2017; Samocha et al. 2007; Suita et al. 2015). The efficiency of this BFT is further improved by the appropriate C–N ratio, which was found to reduce the loads of *Vibrio* even at higher stocking density of shrimp (de Souza et al. 2014) while exhibiting higher abundance of beneficial bacteria, including *Bacteroidia*, *Bacteroidales*, *Flavobacteriia*, *Flavobacteriales* and *Mollicutes* (Padeniya et al. 2022). Thus, the optimum C–N ratio plays a vital role in BFT for diverting autotrophic system to a heterotrophic system that supports the growth and reproduction of heterotrophic bacteria (Avnimelech 1999; Browdy et al. 2001; Xu, Morris, and Samocha 2018). In the current study, total bacterial loads were found in an increasing trend (18.40×10^{6} to 355×10^{9} CFU mL^{-1}) with increasing C–N ratios (Table 4) clearly showing the effects of C–N ratios enhancing microbial communities in a biofloc system. Research has demonstrated that bacterial loads increase in higher C–N ratio, but at an optimum ratio, beneficial heterotrophic bacteria dominate over the harmful autotrophic bacteria; the optimum C–N ratio depends on species (Ebeling, Timmons, and Bisogni 2006; Xu, Morris, and Samocha 2016; Yuniasari and Ekasari 2010). The results of this study (increasing bacterial loads with increasing C–N ratio) directly align with the findings of the previous studies.

Haemocyte counts generally indicate the immunity status of crustaceans (an effective immunological index); higher levels of haemocyte counts represent better immunity, whereas lower counts represent poor immunity (Mridul et al. 2024; Rahi et al. 2022; Rahi, Mather, and Hurwood 2021a; Rodrıǵuez and Le Moullac 2000). Crustacean haemocyte cells consist of hyaline, semi-granular and big granular cells that participate in cellular immune reactions (such as phagocytosis) to fight against germs and pathogens (Rahi, Mahmud et al. 2021). Previous studies showed that C:N ratio in the biofloc system plays a vital role in enhancing the immunity and antioxidant capacity of shrimp (Ekasari et al. 2014; Kumar et al. 2015; Panigrahi et al. 2017; Vazquez et al. 2009; Xu and Pan 2013). Significantly higher haemocyte counts ($p < 0.05$) for *L. vannamei* and *M. rosenbergii* in C10–N (Table 5) clearly indicate the suitability (for improving immunity and production performance) of this ratio for the nursery bi-culture of these two commercially important crustaceans in biofloc system. Thus, optimizing C–N ratio could be an effective way of improving the immunity of shrimps and prawns in a biofloc system for nursery rearing that can subsequently provide higher survival performance.

The C–N ratio in a biofloc system ensures the availability of high-quality feed, which is known to directly influence the biochemical (proximate) composition of shrimp and prawn muscle (Mirzakhani et al. 2019; Tong et al. 2020). In this study, proximate compositions (crude protein, lipid and ash content) of *L. vannamei* and *M. rosenbergii* were found in an increasing trend with increasing C–N ratios (Table 6) indicating the role of differential C–N ratios in manipulating muscle proximate composition of shrimp and prawn. At a higher C–N ratio, floc concentration increases, which subsequently provides higher loads of feed availability for target species (Khanjani and Sharifinia 2022). Increasing the proximate composition of biofloc was gradually elevated to increase C–N ratios (Table 7) to support the findings of previous studies (Gunalan et al. 2013; Hosain, Amin, Kamarudin, Arshad, Romano 2021; Izquierdo et al. 2006; Khanjani, Sharifinia, and Hajirezaee 2020).

In the present experiment, survival rates of *L. vannamei* and *M. rosenbergii* were significantly higher ($p < 0.05$) at C10–N compared to the remaining other ratios (Table 8). Xu, Morris, and Samocha (2016) obtained similar types of findings for growth, production and FCR on *L. vannamei* (higher production at C–N ratio of ≈10). Yuniasari and Ekasari (2010) observed lower survival rates and higher FCR of *L. vannamei* with increasing C–N ratio over 10. Similar findings were also observed for *M. rosenbergii*; higher growth, survival and lower FCR at C–N ratio 10 (Ballester et al. 2017; Kumar et al. 2019; Romano and Zeng 2017). Therefore, it could be suggested that the C–N ratio of 10 is optimum for achieving the best production performance of nursery bi-culture of *L. vannamei* and *M. rosenbergii* in the biofloc system.

5 | Conclusion

This study comprehensively tested the effects of five different C–N ratios on the overall production performance of *L. vannamei* and *M. rosenbergii* for the nursery bi-culture in the biofloc system. The results of this study imply that the C–N ratio directly influenced the growth and survival performance, immunity, body (proximate) composition and water quality parameters. Significantly higher ($p < 0.05$) production performance of both species in terms of growth, immunity, survivability and water quality was obtained for C–N ratio 10:1. Only the proximate compositions of floc as well as *L. vannamei* and *M. rosenbergii* muscle were found to be significantly lower for C–N ratio 10. In addition, the highest benefit–cost ratio (BCR) was obtained at C10–N. Considering all of these facts, it can be concluded that a C–N ratio of 10:1 is optimum for the nursery bi-culture of these two commercially important crustacean species in a biofloc system. This study suggested further research to determine the C–N ratio during the grow-out farming stages could help refine biofloc systems for different developmental phases of these species.

Author Contributions

Md Abdul Halim: conceptualization, data curation, formal analysis, investigation, methodology, software, validation, visualization, writing–original draft. **Aziz Arshad**: conceptualization, funding acquisition, investigation, methodology, project administration, resources, supervision, writing–review and editing. **Dania Aziz**: conceptualization, funding acquisition, investigation, methodology, project administration, resources, supervision, writing–review and editing. **Nur Leena Wong**: conceptualization, methodology, project administration, writing–review & editing. **Murni Karim**: conceptualization, methodology, writing–review and editing. **Muhammad Fadhil Syukri Ismail**: conceptualization, formal analysis, investigation, writing–review and editing. **Md Lifat Rahi**: formal analysis, methodology, software, supervision, writing–review and editing

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

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

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