Biofloc based nutrient dense culture system for nursery and grow-out farming of Pacific white shrimp *Penaeus vannamei* Boone, 1931


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ABSTRACT

The concept of “biofloc technology” is changing the facet of intensive aquaculture with scope to attain high productivity in a sustainable manner. In biofloc, dense heterotrophic bacterial community is developed through C:N ratio manipulation, where the system becomes bacterial dominated rather than algae dominated and takes care of the wastes generated through *in situ* bioremediation. Protein is utilised in two ways; as feed for the shrimp and as microbial floc when the heterotrophic microbes convert the nitrogenous wastes into protein. It also promises a healthy rearing system, which is increasingly identified as one possible solution for disease problems especially those striking at early stages. The purpose of this study was to evaluate the effect of biofloc and periphyton technology (BPT) on the growth and immunomodulatory performance of Pacific white shrimp *Penaeus vannamei* during nursery and grow-out culture. The experimental BPT treatments with three tier substrate system with molasses as carbohydrate (CHO) source were compared with the conventional autotrophic system. The immunomodulation and cumulative percentage mortality upon challenge with pathogenic strain of *Vibrio parahaemolyticus* were assessed in the reared animals. We have successfully demonstrated the BPT based nursery and grow-out systems for *P. vannamei* with the advantage of providing significantly (p<0.05) better growth (27.6% improvement in average body weight, ABW) and feed utilisation (31% improvement in feed conversion ratio, FCR). A production level of 4-4.5 kg m⁻³ of water was achieved through this BPT system registering a significant improvement over the conventional system (p<0.05). The cumulative percentage mortality following pathogen challenge was significantly lower (p<0.05) in the biofloc grown shrimps compared to that of the control group, thus showing better resistance to pathogenic challenge. Furthermore, the biofloc reared shrimp did exhibit significant improvement in non-specific immune response in terms of serum phenoloxidase activity and total haemocyte counts possibly suggesting potential immunostimulatory role of the biofloc associated heterotrophic bacteria. This eco-based technology as revealed through our studies brings substantial improvement in productivity, minimising water requirement, recycling *in situ* nutrients and organic matter in turn improving farm biosecurity, augmentation of natural food, improvement of FCR and better health of the cultured shrimp.

Keywords: Biofloc and periphyton technology, BPT, Heterotrophs, Immunomodulation, Microbial floc, *Penaeus vannamei*

Introduction

Biofloc and periphyton technology (BPT) is a relatively new biotechnological means to support high density culture, maintain water quality, biosecurity, reduce the need for water exchange, reutilise the feed and reduce the production cost. The flocculated microorganisms produced through this process are denser and tend to settle down at the bottom. By developing dense heterotrophic bacterial community, the system becomes bacteria-dominated rather than algae and takes care of the waste generated in the system through *in situ* bioremediation. Disease outbreaks and their impact on commercial shrimp farming operations during the past two decades has greatly affected the operational management of shrimp farms worldwide. BPT approach promises a healthy rearing system, which is increasingly identified as one possible approach for disease prevention.

As the fish/shrimp ponds are rich in microbial community, the inorganic nitrogen added through the feed can be assimilated by these microorganism and converted into microbial protein through an adjustment of C:N ratio. The biofloc principle combines the removal of nutrients from the water with the production of microbial biomass, which can be used by the cultured species *in situ* as additional food source. The optimum C:N ratio in an aquaculture system can be maintained (C:N ratio of 15:1 is optimal for biofloc production) by adding different locally available cheap carbon sources and/or reducing protein percentage in feed. Under optimum C:N ratio, inorganic nitrogen is immobilised...
into bacterial cell while organic substrates are metabolised. In a typical brackishwater pond, only 20-25% of feed protein is utilised by the fish/shrimp, rest of which goes as waste in the form of ammonia and other metabolites, organic N in faeces and feed residue.

Biofloc technology has recently gained attention as a sustainable method to maintain water quality with the added value of producing proteinaceous feed in situ (Crab et al., 2012). In addition to organic nitrogenous waste, ammonium will be converted into bacterial biomass if C:N ratio is balanced at 10-15:1 (Schneider et al., 2005). The cell walls of the microbial constituent of this biofloc, such as bacterial lipopolysaccharide, peptidoglycan and β-1, 3-glucans, stimulate non-specific immunity of fish/shrimp (Panigrahi et al., 2007, 2009). The growth rate and microbial biomass yield per unit substrate of heterotrophs are higher than that of nitrifying bacteria, thus making many fungi increase in heterotrophic bacteria (Hargreaves, 2006). Several studies including that of ours (Arnold et al., 2009; Panigrahi et al., 2014) indicated that biofloc with periphyton systems increased growth, survival and protective response, while also contributing to more favourable water quality.

As BPT promises a healthy rearing system, it is increasingly identified as one possible solution for disease problems especially for diseases striking at early stages (early mortality syndrome (EMS)/acute hepatopancreatic necrosis disease (AHPND) through introduction of biofloc based nursery phases. However, many questions remain unanswered regarding biofloc based mechanisms of antibacterial/antiviral immune responses in penaeid shrimp. The purpose of this study was to evaluate the effect of antibacterial/antiviral immune responses in penaeid shrimp Penaeus vannamei during nursery and grow-out culture.

Materials and methods

Experimental design

A 130 days experiment was conducted using juveniles of P. vannamei during April, 2014 to July, 2014 at Muthukadu Experimental Station, ICAR-Central Institute of Brackishwater Aquaculture (ICAR-CIBA), Chennai, Tamil Nadu, India. The two treatments (Control and BPT) in duplicates were randomly distributed into 4 cement tanks (7.5x1.7x1.1m, 15 m² bottom area, 15000 l capacity,) with 8 compartments. Seawater (previously chlorinated) was filled in all the experimental units. Agricultural lime (CaCO₃) was applied to all the tanks at 200 kg ha⁻¹. Tanks were fertilised with inorganic fertilisers, urea (150 kg ha⁻¹) and single super phosphate, SSP (150 kg ha⁻¹) on the first day. Inorganic fertilisers were applied initially at 2-3 days intervals in split doses. Biofloc was generated by using a mix of yeast, molasses, probiotics (Bacillus subtilis, 5.4 x 10⁹ CFU ml⁻¹), wheat flour, rice bran and pelleted feed mixed/dissolved in autoclaved seawater (50 l), aerated overnight (24 h) and applied uniformly on the third day in all the treatment tanks for development of autotrophs. C:N ratio was maintained followed by the method of Avnimelech (1999) for transition in to heterotrophic system.

Experimental diet preparation

Formulated pellet feed containing 40% of crude protein were used for supplementary feeding in both control and treatment groups. Details of ingredients and proximate composition of the feed are given in Table 1. The coarse ingredients were powdered in a two stage hammer mill and micropulveriser and then passed through 300 µm mesh screen. All the ingredients including liquid ingredients were mixed in a batch mixer as per Table 1. The mixed mash was pelleted in a Ring-Die pellet mill at 16% moisture, at 95°C under steam conditioning.

Biofloc management, shrimp stocking and feeding

Out of the 8 cement compartments, 2 tanks were stocked with juvenile P. vannamei (1.48 ± 0.4 g) at a density of 150 nos. m⁻³. Daily feeding started at 10% of body weight, which was gradually reduced to 3% by the end of experiment. The

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Feed (Control, BPT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein base¹</td>
<td>70</td>
</tr>
<tr>
<td>Carbohydrate base²</td>
<td>24</td>
</tr>
<tr>
<td>Fish oil</td>
<td>2</td>
</tr>
<tr>
<td>Lecithin</td>
<td>1</td>
</tr>
<tr>
<td>Vitamin and mineral mix³</td>
<td>2</td>
</tr>
<tr>
<td>Binder⁴</td>
<td>1</td>
</tr>
</tbody>
</table>

Proximate composition

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>9.49</td>
</tr>
<tr>
<td>Crude protein</td>
<td>39.56</td>
</tr>
<tr>
<td>Ether extract</td>
<td>6.72</td>
</tr>
<tr>
<td>Crude fibre</td>
<td>3.52</td>
</tr>
<tr>
<td>Total ash</td>
<td>12.42</td>
</tr>
<tr>
<td>NFE²</td>
<td>28.29</td>
</tr>
</tbody>
</table>

¹Protein base: Fish meal: Acetes sp.: Soya cake: Gingelly oil cake in the ratio 4:2:3:1
²Carbohydrate base: Wheat: Broken rice: Maida in the ratio 4:2:3
³Vitamins (mg kg⁻¹): Vitamin A 20.0, Vitamin D 4.0, Vitamin E 120.0, Vitamin K 60.0, Choline chloride 6000.0, Thiamine 180.0, Riboflavin 240.0, Pyridoxine 180.0, Niacin 1080.0, Pantothenic acid 720.0, Biotin 2.0, Folic acid 30.0, Vitamin B12 0.150 Inositol 1500.0, Vitamin C 9000.0. Minerals (g kg⁻¹): CaCO₃, 28.0, K₂SO₄, 10.0, MgSO₄ 12.5, CuSO₄ 0.2, FeCl₂ 0.5, MnSO₄ 0.5, KI 0.01, ZnSO₄ 1.0, CoSO₄ 0.01, Cr₂O₃ 0.05, Bread flour 7.14
⁴Poly Methylol Carbamide.
⁵Nitrogen free extract calculated by difference = 100 -(moisture%+Crude protein%+Crude fibre%+Ether extract% + Total ash%)
shrimp were fed with crumbles of 500-800 µ during the first 15 days followed by 1.8 mm pellets and in the last 30 days with 2.0 mm pellets. Feed was distributed equally to shrimps in all the experimental units, thrice daily at 06 00, 11 00 and 18 00 hrs initially for 2 months followed by one additional feeding ration at 22 00 hrs up to harvest. A uniform feeding regime was followed for direct comparison of water quality parameters and microbial dynamics in the experimental tanks. The feeding ration were calculated based on the shrimp biomass in the treatments.

Pre-weighed molasses was mixed in a beaker and uniformly distributed over the tank surface directly after feed application at 11 00 and 18 00 hrs. Since molasses contained 28% carbon, 1.0 g molasses was added for each 1.0 g of feed in the experimental groups. To increase the C: N ratio to 10:1, molasses was added assuming that 10 g of carbon is required to convert 1 g of total ammonia nitrogen, generated from excreta and uneaten feed, into bacterial biomass (Avnimelech, 1999). Continuous aeration and agitation was provided employing one 5 hp blower passing through sand stones aerator, fixed 10 cm above the soil bottom layer, with capacity of 7.5 m³ air per tank per min. In the biofloc treatment tanks, minimal water exchange principle was followed throughout the experimental period.

**Proximate composition of experimental diets**

The proximate composition of experimental diet was determined following the standard methods of AOAC (1995). The moisture content was determined by drying at 105°C to a constant weight and the difference in weight of the sample indicated the moisture content. Nitrogen content was estimated by Kjeldahl (Kelplus, DXVA, Pelican equipments, India) method and crude protein was calculated by multiplying nitrogen percentage by 6.25. Crude lipid was determined by the solvent extraction method by Soxtec system (Soxtec system, SCS-6, Pelican equipments, India) using diethyl ether (boiling point, 40-60°C). The growth performance was assessed in terms of length gain (mm), average weight gain (AWG) (g week⁻¹), final biomass (g m⁻²), survival (%) and specific growth rate (SGR), whereas, feed conversion ratio (FCR) and protein efficiency ratio (PER) were recorded to assess shrimp performance in terms of feed utilisation. All the shrimps were sampled thrice a month for measuring the above parameters. At the end of the experiment, the above mentioned indicators were estimated in addition to final yield (kg ha⁻¹). The growth parameters were calculated using the following formulae:

**Assessment of water quality parameters**

Water quality of the experimental systems was checked at weekly intervals at 09 00 hrs. Water parameters such as temperature (mercury thermometer), pH (pH-Scan-Eutech instruments, Singapore), salinity (hand refractometer), total ammonia nitrogen, TAN (Phenol hypochlorite method), NO₃-N, NO₂-N, phosphate-P (PO₄-P), total alkalinity, turbidity and dissolved oxygen were analysed following APHA (1998). Total suspended solid (TSS) was determined every fifteen days’ interval (APHA, 1998). Biofloc volume was quantified employing Imhoff cone on daily basis to understand the dynamics of biofloc generation and to adopt control measures in case of excess biofloc generation if any.

**Estimation of microbial biomass**

Total heterotrophic bacterial count and Vibrio count of water and soil samples were determined at 10 days interval up to 130 days of the experiment. Water samples were collected in sterile poly-propylene bottles from the centre of the tank. The samples were maintained at 4°C and immediately brought to the laboratory. Two hundred ml of water sample was homogenised in a kitchen blender at 12000 rpm for 30 sec. Subsequently, tenfold serial dilution was made in normal saline solution (NSS) and 0.1 ml of appropriate dilutions were plated in duplicates on Zobell marine agar containing 1% NaCl (w/v) for total count and on thiosulfate citrate bile salts sucrose agar (TCBS agar) for Vibrio count. Plates were incubated at room temperature for 48 h and colony in the range of 30 to 300 were counted and expressed as bacterial colony forming unit (cfu).

**Estimation of growth and production parameters**

The growth performance was assessed in terms of length gain (mm), average weight gain (AWG) (g week⁻¹), final biomass (g m⁻²), survival (%) and specific growth rate (SGR), whereas, feed conversion ratio (FCR) and protein efficiency ratio (PER) were recorded to assess shrimp performance in terms of feed utilisation. All the shrimps were sampled thrice a month for measuring the above parameters. At the end of the experiment, the above mentioned indicators were estimated in addition to final yield (kg ha⁻¹). The growth parameters were calculated using the following formulae:

\[
\text{Weight gain} = \frac{\text{Final weight (g)} - \text{Initial weight (g)}}{\text{Initial weight (mg)}} \\
\text{Length gain} = \frac{\text{Final length (mm)} - \text{Initial length (mm)}}{\text{Initial length (mm)}} \\
\text{Specific growth rate} (SGR) (%) = \frac{\ln \text{final weight} - \ln \text{initial weight}}{\text{Days of culture} \times 100} \\
\text{Average daily growth (ADG)} (mg day⁻¹) = \frac{\text{Final weight (mg)} - \text{Initial weight (mg)}}{\text{Experimental duration (days)}}
\]
Shrimp no. at the end of experiment \( \times 100 \)
Shrimp no. at the beginning of experiment

\[ \text{FCR} = \frac{\text{Feed applied}}{\text{Body weight gain}} \]

\[ \text{FER} = \frac{\text{Weight gain}}{\text{Feed intake}} \]

\[ \text{PER} = \frac{\text{Net weight gain}}{\text{Protein in feed applied}} \]

**Immune parameters**

The haemolymph from sampled shrimp was collected using a 2 ml syringe (21G) with or without anticoagulant saline, from the ventral sinus of the first abdominal segment, as per the methodology for the required immune parameters. The anticoagulant saline solution was prepared by mixing NaCl (340 mm), KCl (13 mm), MgSO4 (11 mm), MgCl2 (10 mm), NaH2PO4 (0.3 mm) and glucose (1.6 mm) in 100 ml distilled water, pH of the solution was adjusted to 7.8 using NaHCO3. The haemocytes count was carried out as per the methodology of Wootten et al. (2003) and Soderhall (1982). Similarly, phenoloxidase activity was determined as per the methods of Soderhall and Cerenius (1992).

**Challenge trial**

Experimental infection was carried out with permission from the Ethical Committee for Animal Experiments of ICAR-CIBA, following and the guidelines of OIE was followed. The biofloc treated and control intermoult shrimps of 12-15 g ABW were stocked into 100 l FRP tanks, with 20 animals in each tank in triplicate. The levels of pH, salinity and oxygen were maintained at optimum levels and the biofloc environment was maintained. A pathogenic strain of *Vibrio parahaemolyticus* (MTCC 451) sourced from the Institute of Microbial Type culture collections (IMTCC), Chandigarh, India was cultured overnight in Zobell marine broth 2216 (Himedia, India). Twenty millilitre ml of a suspension of *V. parahaemolyticus* containing \( 1.21 \times 10^8 \) cells ml\(^{-1} \) were introduced into the tank. All the shrimps were closely monitored following experimental infection for mortality. No water was exchanged during the experimental trial. During challenge tests, water quality parameters, shrimp survival and cumulative numbers of dead shrimps were recorded every day.

**Statistical analysis**

The data were statistically analysed by statistical package SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). Before all analysis data were analysed for normality by probability plots and Kolmogorov-Smirnov test and for homogeneity of variances by Levene’s test. One way ANOVA was used to determine the significance of each parameter among different treatments. If a main effect was significant, the ANOVA was followed by Tukey’s test. Level of significance was determined at 99 and 95% probability levels.

### Results

**Water quality parameters**

The water quality parameters of the experimental tanks recorded during the study are presented in Table 2. Temperature and salinity in the experimental tanks ranged from 28.5-31.2 \(^0\)C and 28-30 ppt, respectively during the study period. Biofloc treatments showed significant effect (p<0.01) on pH reduction irrespective of protein level in feed. Alkalinity during the entire culture period showed non-significant reduction (p>0.05) in BPT (18.03±1.89%) than control group. There was a significant decrease (p<0.05) in the ammonia concentration in the BPT groups (0.313 ± 0.05) with molasses supplementation compared to the conventional group (0.743±0.02). Carbohydrate supplementation significantly (p<0.05) reduced TAN levels in BPT group (42.09±5.3%) when compared to that of control group. The mean value of TAN and nitrite at 15 days interval are given in Fig. 1 and 2, respectively.

Similarly, nitrite-N, nitrate-N values were significantly reduced in biofloc with periphyton groups and were

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>BPT-40</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAN (ppm)</td>
<td>0.74±0.02(^a)</td>
<td>0.31±0.05(^b)</td>
</tr>
<tr>
<td>NO(_2)-N (ppm)</td>
<td>0.50±0.02(^a)</td>
<td>0.14±0.02(^b)</td>
</tr>
<tr>
<td>NO(_3)-N (ppm)</td>
<td>0.29±0.10(^a)</td>
<td>0.20±0.05(^b)</td>
</tr>
<tr>
<td>PO(_4)-P (ppm)</td>
<td>0.15±0.09(^a)</td>
<td>0.23±0.13(^b)</td>
</tr>
<tr>
<td>Alkalinity (ppm)</td>
<td>197.0±7.8(^a)</td>
<td>168.8±4.6(^b)</td>
</tr>
<tr>
<td>Floc volume (ml)</td>
<td>5.13±1.2(^a)</td>
<td>18.6±2.2(^b)</td>
</tr>
<tr>
<td>TSS (ppm)</td>
<td>167.9±3.3(^a)</td>
<td>575.8±3.9(^b)</td>
</tr>
<tr>
<td>Turbidity (NTU)</td>
<td>16.67±2.58(^a)</td>
<td>26.98±3.68(^b)</td>
</tr>
</tbody>
</table>

Each value represents mean±S.D. Values in the same row with different superscripts are significantly different (p<0.05).

![Fig. 1. Mean values of TAN in 15 days time intervals of control and biofloc tanks](image-url)
34.05±2.2% and 40.5±9.6%, respectively. But, Phosphate-phosphorous level was found to be more in BPT treatments (43.4±4.4%) compared to that of the control group.

Quantitative analysis of biofloc

Microscopic observation of bio-floc revealed that it comprised certain particulate and flocculated microbes, rotifers, copepods; microalgae, protozoan communities like ciliates and detritus. The quantity of biofloc developed was measured using Imhoff flask in terms of turbidity and is presented as monthly average in Table 2. In BPT system with carbohydrate supplementation, turbidity (26.98±3.68 NTU) was significantly higher (p<0.05) compared to control group (16.67±2.58 NTU). Similarly, supplementation of molasses resulted in significantly higher total suspended solids (TSS) in BPT (74.7±5.0%) when compared to that of control group (Fig. 3). Bio-floc volume was significantly (p<0.05) higher in BPT (18.75±3.9 ml) tanks compared to control (5.13±1.2 ml) (Fig. 4).

Microbial analysis

Carbohydrate supplementation significantly increased the total heterotrophic bacterial (THB) count of water in BPT (82.9±4.4%) system as compared with control groups. As the culture progressed, the increasing biomass had significant effect (p<0.01) over total microbial load with higher levels recorded in BPT compared with control. Similarly, carbohydrate supplementation had significant effect on total Vibrio count (TVC) in water (p<0.01). TVC levels were greatly reduced in BPT water (91.80±0.3%) whereas in control group, it was higher. The carbohydrate supplementation resulted in increase of total plate count (TPC, 71.7±3.3%) in water of BPT groups. The proportion of Vibrio count to total heterotrophic bacterial count (V/T) was lower in the biofloc groups compared to that of the control group. In spite of increase in Vibrio load, the V/T ratio was non-significantly (p>0.05) lower in the BPT groups. The V/T ratio was lower in the BPT water (11.2±1.2%) as compared to that of control (96.5±12.8%).

Growth performance

The data on growth performance of BPT and control groups is presented in Table 3. The average body weight was significantly (p<0.01) higher in the bio-floc treated groups (38.15±3.36) when compared to control group (29.91±3.58) (Fig. 5).

The improvement in average body weight in the biofloc group was 27.57% over the control group. The survival rate of the BPT group (84.13±0.1%) was also significantly higher (p<0.05) compared to that of the control group (70.31±0.1%) registering an improvement of 19.6%. Production in the biofloc treatments (43.4±19.6 t ha⁻¹) was significantly higher compared to that of the control (28.42±1.2 t ha⁻¹) (Table 3). BPT groups also showed a significant increase (32.6 to 52.6%) in productivity when compared with that of the
Table 3. Growth performance including weight gain, FER and PER of shrimp

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Control</th>
<th>BPT</th>
<th>Significance</th>
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</thead>
<tbody>
<tr>
<td>Initial weight (g)</td>
<td>1.91±0.48a</td>
<td>2.41±0.59b</td>
<td>**</td>
</tr>
<tr>
<td>Final weight (g)</td>
<td>29.91±3.58a</td>
<td>38.15±3.66b</td>
<td>**</td>
</tr>
<tr>
<td>Weight gain (g)</td>
<td>17.49a</td>
<td>22.34a</td>
<td>**</td>
</tr>
<tr>
<td>Survival rate (%)</td>
<td>70.31±3.3a</td>
<td>84.13±3.86a</td>
<td>*</td>
</tr>
<tr>
<td>SGR (%)</td>
<td>2.11a</td>
<td>2.13a</td>
<td>*</td>
</tr>
<tr>
<td>FCR</td>
<td>2.07a</td>
<td>1.58b</td>
<td>*</td>
</tr>
<tr>
<td>FER</td>
<td>0.48a</td>
<td>0.63b</td>
<td>*</td>
</tr>
<tr>
<td>PER</td>
<td>1.21a</td>
<td>1.58b</td>
<td>*</td>
</tr>
<tr>
<td>Productivity (t ha⁻¹)</td>
<td>28.42 ± 1.2a</td>
<td>43.4± 19.6b</td>
<td>**</td>
</tr>
</tbody>
</table>

Each value represents mean ± S.D. Values in the same row with different superscripts are significantly different (p<0.05)
*significant at p<0.05; **significant at p<0.01

Fig. 5. Mean values of ABW in control and BPT based shrimp culture system

control non-biofloc group. The FCR recorded in the group (1.58±0.2) was lower compared to control group (2.07±0.2). There is an improvement of 10 to 31% as reflected in the declining FCR in the biofloc group. Similarly, higher PER and FER of 1.58±0.15 and 0.63±0.12 respectively were observed in BPT compared to control group (1.21±0.4 and 0.48±0.10 respectively). The proximate composition of shrimps from the two experimental groups are presented in Table 4.

Immunological parameters and challenge trial

Total haemocyte count was significantly (p<0.05) higher in BPT reared shrimps (17.7±1.4 x 10⁶ cells ml⁻¹) when compared to control (7.1±0.75 x 10⁶ cells ml⁻¹) (Fig. 6).

Serum phenoloxidase activity (Fig. 7) was found higher in the BPT treated shrimps (0.146±0.009 units min⁻¹ mg protein⁻¹) compared to control shrimps (0.051±0.003 units min⁻¹ mg protein⁻¹). Cumulative percent mortality of conventionally grown shrimps was significantly higher than the BPT group (Fig. 8) when challenged with V. parahaemolyticus.

Table 4. Proximate composition (%) of P. vannamei (as % wet weight basis) cultured in the BPT and non-BPT systems

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Moisture</th>
<th>Crude protein</th>
<th>Ether extract</th>
<th>Crude fibre</th>
<th>Total ash</th>
<th>NFE⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>76.38±0.94</td>
<td>16.40±0.37</td>
<td>1.06±0.02</td>
<td>1.37±0.12</td>
<td>2.61±0.15</td>
<td>2.18±0.93</td>
</tr>
<tr>
<td>BPT</td>
<td>77.18±1.32</td>
<td>16.55±0.12</td>
<td>1.08±0.06</td>
<td>1.42±0.17</td>
<td>2.53±0.14</td>
<td>1.24±1.39</td>
</tr>
</tbody>
</table>

⁺Nitrogen free extract = 100 - (moisture%+Crude protein%+Crude fibre%+Ether extract% + Total ash%)
The challenge trial revealed that the mortality rate was higher in conventionally grown shrimps than those reared in BPT based systems. Mortality of 57.9% was recorded in challenged control shrimps but a significantly lower mortality of 37.5% was observed in shrimps reared in biofloc based systems, eight days post-challenge.

**Discussion**

Water quality parameters play an important role in aquaculture. The temperature and salinity which are important parameters (Chen et al., 1990, Hariati et al., 1996), did not vary significantly in the treatments in the present study. The TAN level was observed to be higher in the control tanks as compared to the treatment tanks. The findings are in agreement with Hari et al. (2004); Avnimelech and Mokady (1988); Avnimelech et al. (1989) and Avnimelech (1999) who reported that addition of carbohydrate to the production systems reduces the TAN concentration through immobilisation by bacterial biomass. It has been reported that fish in a pond assimilate only 15-30% of the nitrogen added in the feed (Acosta-Nassar et al., 1994; Gross et al., 2000; Davenport et al., 2003), the remainder being lost to the system as ammonia and organic N in faeces and feed residue, which also undergoes decomposition and eventually produces ammonia. Therefore, higher dietary protein levels lead to significantly higher TAN and NO$_2$-N concentrations in the water column. Li and Lovell (1992) reported that the ammonia concentration increased with increasing dietary protein concentration and protein feeding rate. Similarly, in this experiment, TAN, NO$_3$-N and NO$_2$-N levels decreased in the biofloc treated tanks because of the addition of carbon sources whereas control tanks recorded comparatively high values. Also, it was reported earlier that zero water exchange ponds using carbon enable to control the accumulation of inorganic nitrogen through a balanced ratio of carbon to nitrogen of the feed (Avnimelech et al., 1989, 1992, 1994; Avnimelech, 1998, 1999). In addition, Stuart et al. (2009) raised tiger shrimp *P. monodon* in zero water exchange model using a daily carbon source (tapioca powder) to promote the microbial community and improve water quality. Xu et al. (2015) reported that abundance of algae and heterotrophic bacterial population increase phosphate content in the biofloc treatment groups. In the present study, total phosphate was significantly higher in BPT compared to control. According to Jarrett et al. (1993), high primary production would lead to decline in alkalinity. Thus, maintenance of optimum alkalinity through liming is very important since calcium is also crucial for shrimp growth and moulting (Tseng, 2009). Our study revealed that the total alkalinity levels were significantly low in the BPT groups than control. Furthermore, present study revealed that the levels of TSS, turbidity and biofloc volume showed significant difference between BPT and control groups. This can be converted from feed (protein as nitrogen) and carbon source, which subsequently increased bacterial/microbial population in the biofloc systems (Huchette et al., 2000; Azim, 2001).

In the biofloc, organic matter, zooplankton communities like ciliates, rotifers, copepods and a small amount of autotrophic microalgae were noticed during the present study. Similarly, Ballester et al. (2010) reported that biofloc is composed of attached heterotrophic bacteria, filamentous cyanobacteria, dinoflagellates, ciliates, flagellates and rotifers. Various factors like salinity, light and type of culture system affects the microbial composition of biofloc. For example, Ju et al. (2008a) reported the dominance of algal communities over bacterial biomass in flocs collected from outdoor shrimp culture units. Lower dominance of autotrophic community noticed in the present study might be due to lack of direct sunlight in the biofloc indoor production facility (Shyne Anand et al., 2014).

Many studies have demonstrated beneficial effects of biofloc on shrimp culture (Wasielesky et al., 2006; Ballester et al., 2010; Ray et al., 2011; Haslun et al., 2012; Xu and Pan, 2012; Zhao et al., 2012). Several researchers suggested that apart from maintaining good and stable water quality, the established biofloc in the culture system can improve growth performance and feed utilisation of different shrimp species viz., *Penaeus monodon* (Arnold et al., 2009), *P. semisulcatus* (Megahed, 2010), *Farfantepenaeus paulensis* (Ballester et al., 2010), *P. vannamei* (Xu and Pan, 2012) and *Marsupenaeus japonicus* (Zhao et al., 2012).

Survival rate was significantly higher in biofloc treatments compared to control, indicating no increased stress due to the presence of biofloc (Megahed et al., 2014). Higher survival and improved growth of the shrimp in all the biofloc treatments (Xu and Pan, 2012) can also, in turn, support the view that the shrimp grew in a healthy condition in biofloc-based tanks with carbohydrate addition.
The presence of biofloc helped to improve shrimp growth, survival rate and the culture productivity in the present study. The findings corroborate the results of other studies that show the positive effect of biofloc on production indices for the culture of post-larvae and juvenile shrimp (Arnold et al., 2009; Audelo-Naranjo et al., 2010; Lezama-Cervantes and Paniagua-Michel, 2010; Zhang, 2011; Viau et al., 2012). Reduction of dietary protein level without affecting growth performance of cultured shrimp in the presence of biofloc has been reported by several authors (Hari et al., 2004; Ballester et al., 2010). This is in accordance with earlier investigation by several authors (Arnold et al., 2009; Asaduzzaman et al., 2009, 2010) which revealed that the growth was significantly greater when carbon was applied in shrimp culture ponds with zero water exchange model. Inclusion of biofloc as a dietary ingredient in shrimp diet was found to improve the growth performance of P. vannamei (Ju et al., 2008b; Kuhn et al., 2009, 2010).

The present study revealed that the total heterotrophic bacterial counts was significantly higher in the BPT tank water as compared to control tanks. Appropriate carbohydrate addition could result in heterotrophic bacteria effectively assimilating the TAN and NO₃-N to synthesise bacterial protein and new cells (Kirchman and Keil, 1990; Avnimelech, 1999; Touratier et al., 1999; Wang and Yin, 2009). Similar results were also found in Macrobrachium. rosenbergii culture system by Asaduzzaman et al. (2008). Massive bioflocs resulted from active bacterial metabolism and growth under the carbon addition (Burford et al., 2004; Azim and Little, 2008; Crab et al., 2009) might also provide appropriate substratum for the colonisation and growth of autotrophic as well as heterotrophic bacteria. In BPT system, the vibrio counts were recorded low compared to the control. The study revealed that the application of biofloc inoculum leads to abundance of THB and decreased vibrio load in the treated water. It was also effective in controlling Vibrio population and thus helped to improve water quality and subsequently shrimp health. These findings are similar to the observations made by Ravichandran and Jallaluddin (2000), who used Environ-AC @25 kg ha⁻¹ as an initial dosage followed by 10 kg at weekly interval.

Crustaceans, especially, shrimps lack a specific, adaptive immune system and rely entirely on their innate immune mechanisms that include both cellular and humoral responses for defense against pathogens (Vazquez et al., 2009). The circulating hemocytes play a central role in cellular and humoral immune system (Bachere et al., 2004). The circulating haemocyte count has been considered a functional indicator of immune capability (Rodriguez and Le Moullac, 2000). In the present study, shrimps reared in BPT based system had significantly higher (p<0.05) total haemocyte counts compared to control shrimps.

The pro-phenoloxidase (pro-PO) activating system is an important part of immune response in shrimps, which includes recognition of foreign invaders and nonliving entities, activation of a wide range of defence reactions, such as phagocytosis and antibacterial activity, encapsulation and nodule formation (Soderhall, 1982; Soderhall and Cerenius, 1992). In the present study, serum phenoloxidase (PO) activity was significantly higher in shrimps from BPT based culture compared to control group shrimps. BPT systems induce the activation of the host immune system when the disease causative pathogen is in close proximity. Cumulative percent mortality of conventional and BPT group shrimps challenged with V. parahaemolyticus showed significant difference. BPT are often viewed as a mechanism that provides shrimp with pattern recognition that ultimately lead to stimulation of non-specific immune system. It is also hypothesised that the constituents of bacterial cell walls trigger the non-specific immune system in shrimps.

This biofloc based technology as revealed through our studies brings out the advantages like recycling nutrients/organic matter, improving water quality through in situ bioremediation, providing stress-free environment, augmentation of natural food, improvement of growth, immunity and FCR of the cultured shrimps. Improving farm biosecurity by exclusion of pathogens, avoidance of chemotherapeutics, antibiotics with negligible environmental impact are among the other features of this system. Obviously, this upcoming technology will ensure high productivity though a sustainable and environmental friendly approach.

References


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