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Research Article

Environmental amelioration in biofloc based rearing system of white leg shrimp (*Litopenaeus vannamei*) in West Bengal, India

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Abstract – The potential of biofloc media in *in-situ* environmental amelioration mediated through the removal of nitrogenous metabolites, reduce the dominance of disease-causing *Vibrio* to beneficial *Bacillus* community and reduction of costly commercial feed was investigated in white leg shellfish (*Litopenaeus vannamei*) farming. Three treatments viz. biofloc without neem + commercial feed (B), biofloc with neem +commercial feed (NB), only commercial feed without biofloc (F)and, one control (C) withno biofloc and no feed in triplicate was applied with a stocking density of 60 PL-20 m^{-3} in outdoor earthen ponds (1000 m^3). Two biofloc media (C:N = 15–10:1) with and without neem leave extract were applied with reduction of feed supplement from 50% to 100%. The significant difference was observed in final body weight (33.82 ± 0.18 g) in neem based biofloc media than the other tested shrimp with the survival percentage above 87%. Superiority of biofloc based rearing system over the traditional feed based one was established as live weight gain was 28.48–137% more with a reduction of feed conversion ratio by 7.60% to 8.18% in the former. Moreover, feed cost was reduced both in B (8.1%) and NB (8.77%) compared to conventional feed −based culture system (F). The nutrient profile of floc and shrimp also improved as higher protein content was recorded in floc (18.65–19.63%) and shrimp (24.58–26.29%). The development of biofloc within the culture system resulted in cumulative increase of *Bacillus* population with concomitant decrease of luminous *Vibrio* population. The findings of the study strongly indicated that biofloc technology could be a potential tool of ecosystem approach towards *in-situ* environmental amelioration in shrimp farming ponds with substantial reduction of cost towards feed, aqua chemicals used for disease and environmental health management and other operational cost like water exchange.

Keywords: Biofloc / *Azadirachta indica* / *Litopenaeus vannamei* / Feed conversion ratio / Heterotrophic bacteria / *Luminous vibrio* / Probiotic bacteria

1 Introduction

White leg shrimp (*Litopenaeus vannamei*) is an economically important shrimp species for inland coastal aquaculture with a global production of 4966.20 thousand tonnes in 2018 which accounts for 52.9% share of the total major species production in aquaculture (FAO, 2020). The culture of *L. vannamei* is becoming increasingly popular throughout the globebecause of its rapidgrowth rate, high survival and wide tolerance to diseases and environmentalstressors such as temperature and salinity than other commercially cultured shrimp species (Moss et al., 2007). However, farming of white leg shrimp has largely been affected in recent times primarily out of disease outbreaks in culture ponds leading to farming setbacks with increased use of antibiotics (Luckstadt, 2020). Eventually, this sector is also facing challenges of water quality degradation, disease problems, improper nutrient management and poor growth performance due to lack of understanding and application of ecosystem approach (Brugère et al., 2018).

Though, several economically intensive technologies are available, eco-friendly biofloc technology is the most effective and sustainable method in mitigating such problems in

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aquaculture (Ahmed et al., 2017). As biofloc alone is insufficient to guarantee the level of growth and survival required for intensive aquaculture practices, it must be amalgamated with application of formulated feed to satisfy the nutritional requirements of the culture species. Biofloc along with formulated feed was able to enhance the fish growth performance and feed utilization efficiency (Meritha et al., 2018). Also, in polyculture of Indian major carps, biofloc system can enhance culture efficiency and fish production (Deb et al., 2020). Different aspects of biofloc technology in shrimp culture have been investigated in coastal aquaculture system (Avnimelech, 2009; Emerenciano et al., 2012; Xu and Pan, 2012; Hamidoghli et al., 2020; Olier et al., 2020; Fleckenstein et al., 2020).

Biofloc technology has been described as a self-sustaining biotechnologically sound in situ auto-remediation system in which nutrients could be continuously recycled and reused. It can enhance higher fish production up to two-fold increase through effective utilization of water and land resources. It is evident that carbon and nitrogen (C:N) ratio in water influences the development of biofloc which have affected on water quality as wellas the nitrogen and phosphorous transform dynamics and shrimp growth rate (Ray et al., 2011; de Lorenzo et al., 2016; Xu et al., 2016). Total dissolved solids concentration in water also can play a major role in biofloc system, as solids concentration above 800 mg l^{-1} can lower shrimp growth (Schveitzer et al., 2013).

The application of external carbon sources (molasses, jaggery, glucose, etc.) aids to maintain suitable carbon and nitrogen (C:N) ratio within 10−20:1 to accelerate growth and reproduction of heterotrophic microbial populations for assimilation of the organic nitrogenous metabolites to reduce the ammonia load in minimum or zero water exchange biofloc system (Emerenciano et al., 2012; Da Silva et al., 2013; Zao et al., 2014; Panigrahi et al., 2018). Biofloc system has the great potential in removal nitrite and total ammonia nitrogen load from fish culture system (Deb et al., 2017). Dinda et al. (2019) reported that biofloc favored phosphorus mineralization and became limited in maintaining the balance between available nitrogen: phosphorus (N:P) beyond 0.065 mg L^{-1}. Biofloc contains rich bioactive compounds which increase the tolerance to stress and help in activating the antioxidant activity in shrimp (Babin et al., 2010) through probiotic activity (Mandal and Das, 2018). The presence of bioactive compounds and probiotic microbes in biofloc improve health and immune system of *Oreochromis niloticus* larvae and *Litopenaeus vannamei* juvenile (Xu and Pan, 2013; Ekasari et al., 2015). Mandal and Das (2018) reported that the probiotic effect of *Bacillus cereus* and *Lactobacillus* sp. in biofloc caused the reduction if bioluminescence problem caused by pathogenic *Vibrio* sp. in grow-out shrimp culture. Biofloc can also act as a supplementary food to the culture species through recycling the unutilized fraction of conventional feed (Xu and Pan, 2014). Biofloc could be considered as good protein source for shrimp and tilapia due to varied level of amino acid composition and sufficient lipid source for *Pangasius* sp. (Meritha et al., 2018). Panjaitan (2004) reported that 30% reduction in conventional feed was possible in shrimp culture with biofloc.

The gram-negative bioluminescent *Vibrio* species are responsible for causing luminescent vibriosis with mass mortalities in penaeid shrimp (Phuoc et al., 2009). Herbal plants are good source of natural therapeutic antimicrobial agent (Banerjee et al., 2013). All the parts of neem (*Azadirachta indica*) have wide range of pharmacological properties (Farah et al., 2006). Application of different parts of neem in aquaculture to control pathogen and disease (Martinez, 2002) and in the amelioration purpose (Biswas et al., 2002) has gained momentum in the recent years. The presence of a natural bioactive compound, azadirachtin in neem highly influence the phosphatase activity (Gopal et al., 2007) and to some extent inhibit the nitrification process in aquaculture (Das et al., 2018). Crude preparation of neem leaf juice which is widely used by the shrimp farmers in the study area has been reported as an antibacterial agent for inhibition of vibriosis in shrimp (Banerjee et al., 2013).

Therefore, the present study was aimed toevaluate the potential of biofloc technologyas a tool in the in situ environmental amelioration process of nitrogenous metabolites in the culture units, in maintaining healthy ecosystem by reducing disease-causing *Vibrio* population and to assess the production performance of white leg shellfish (*Litopenaeus vannamei*) with reduction of the quantity of commercial feed use in thepond basedproduction of white leg shellfish (*Litopenaeus vannamei*).

2 Materials and methods

2.1 Study area and experimental set ups

The biofloc experimentation on *L. vannamei* farming was carried out at Rasulpur (Latitude 21° 39' 20" N and Longitude 87° 20' 50" E), West Bengal, India in twelve outdoor earthen ponds (1000 m^3; area: 1000m^2; water depth: 1m) randomly grouped into four batches which were allotted to three treatments viz. B (biofloc without neem + commercial feed), NB (biofloc with neem + commercial feed), F (commercial feed) and one control, C (no biofloc, no commercial feed). All the ponds were supported with high aeration facility. Four paddle wheel aerators (2 hp each) were placed in each pond. Water (salinity 15) was filled up to 1m pond depth and was chlorinated by applying bleaching powder at the rate of 150 kg ha^{-1} m^{-1} depth. After 5 days of chlorination, liming was done with agricultural lime (300 kg ha^{-1}) followed by fertilization after seven days of liming (single super phosphate 20 kg ha^{-1} and urea 40 kg ha^{-1}). When the water colour turned bluish green indicating development of planktonic organisms, ponds were stocked with 20 days oldhealthy PL-20 of *L. vannamei* (15−16 mg; 12−15 mm) at density of 60 PL m^{-3}. Commercial floating shrimp feed (Charoen Pokphand −CP, India; crude protein-42.02%) was used for feeding the *L. vannamei* at the rate of 6% body weight day^{-1}, divided into three equal installments (10 AM, 2 PM and 5 PM) in the respective treatments during the first 30 days and gradually reduced thereafter to 4% body weight day^{-1}. Along with feeding, treatment B and NB were applied with without neem and with neem supplemented media, respectively, for in-situ development of biofloc. Treatment F was not applied with either of the medium and in the control treatment the shrimps were supported with natural food alone. The culture progressed with zero water exchange except periodic addition of chlorinated water to make up the water loss due to sampling,

Table 1. Composition of biofloc media preparation for use in pond culture of *L. vannamei*.

Ingredients	Biofloc without neem	Biofloc with neem
	Amount	Amount
Boiled neem leaves extract (l)	0.00	20.00
Boiled rice water (l)	30.00	0.00
Molasses (l)	20.00	20.00
Yeast powder (g)	250.00	250.00
Bore well water (l)	10.00	10.00

evaporation and seepage. For such replenishment, water (salinity 15) from an adjacent buffer reservoir was pumped (Sunsun JDP 10000 Model JDG 10000Voltage: DC-24V) to each experimental pond as and when necessary.

2.2 Preparation of medium

Two different media (without neem and with neem) were prepared through aerobic fermentation in two separate cement cylindrical cisterns (area: 0.15 m^3, depth: 1m; volume: 150 L) supported each with continuous aeration (Tab. 1). Semi liquid molasses was used as main external carbon source and Bakers' Yeast was used as a fermenter as well as probiotic. All the ingredients were weighted and mixed properly with waterand left for 72 hours for fermentation. After which, the colour of the media turned into yellow which indicated that the media were ready to apply. Finally, the slurry from both the cisterns was sieved with a fine cloth and stored in separate tanks respectively. Media were applied at the rate of 10 liters per 1000 m^3 pond at an alternate day (9−10 AM) during the first 50 days of total experiment durationof 105 days. The purpose of the preparation of biofloc medium and its application in the respective experiments was the development of biofloc and heterotrophic microbial populations in the pond culture environment.

2.3 Maintenance of carbon: nitrogen (C:N) ratio

The required amount of carbohydrate (molasses and boiled rice water) added to the treatment ponds to achieve the required C:N ratio was calculated according to the formula of Avnimelech (2009). C:N input ratios were calculated based on the carbon-nitrogen contents of the feed and the carbon content of the molasses. The commercial pelletfeed (CP Blanca, CPF (India) Pvt. Ltd.; 42.02% crude protein) procured from MS Chintamoni Mandal, Contai, Midnapur (East) for *L. vannamei* had a calculated C:N ratio of 10:1, assuming the feed had 50% carbon and 6.72% of nitrogen. Application of molasses as external carbon source to one gram of feed mainly depends on the nitrogen assimilation and assumption in the system (Panigrahi et al., 2018). In the present study, 1.36 ml and 0.68 ml molasses per gram feed was added to maintain C: N ratio 15:1 and10:1 in the treatments respectively. As the stocking biomass was 930 g, with a feeding rate at 6% body weight 56 g of feed was required for each culture pond, 76.16 ml molasses was added for achieving C: N ratio of 15:1. With the increasing biomass, the amount of feed and molasses were adjusted so as to maintain C:N ratio at 15:1 up to 30 days of culture for biofloc development and maturation after which the C:N ratio was lowered to 10:1 by reducing the input of molasses for the stable stage of biofloc. Diluted molasses was applied in respective treatments daily (10.30 AM) with higher aeration to facilitate uniform mixing in water.

2.4 Collection of samples

Collection of water and soil samples were performed aseptically in sterilized polypropylene bottles (200 ml) at 15 days intervals from grow out ponds of *L. vannamei* for physico-chemical and bacteriological analyses following the methods described by Arulmoorthy et al. (2014). Soil samples were collected using mini hand grab sampler. The samples were pooled together by thorough mixing, air dried, pulverized with pestle and mortar, sieved (150 μm mesh) and stored in labeled polythene packets foranalyses.Biofloc samples were collected using Imhoff cone (1 liter capacity) (Faizullah et al., 2015) once in two weeks intervals from each of the experimental ponds and the volume of the floc plug accumulated on the bottom of cone after 10–15 minutes for settlement was used for volume and qualitative determination (Avnimelech and Kochba, 2009). One ml of biofloc sample was analyzed under a binocular microscope and composition of biofloc was identified as par the keys of Edmondson (1959) and Patterson (1992). Proximate composition of biofloc was analyzed at the end of the experiment from the respective treatments.The sample of biofloc was filtered through 100-μm mesh and a subsample of the concentrate flocs were centrifuged at 6000 rpm, dried in hot air oven at 45°C, kept in airtight containers and stored in refrigerator for proximate composition analyses.

2.5 Analyses of samples

2.5.1 Physico-chemical parameters

Surface water temperature and pH (pH HI98127), salinity (Brix RHB-32ATC) and total dissolved solids (TDS) (Eutech-N374) of water samples were measured on spot. Standard methodologies were followed for estimation of dissolved oxygen (DO), biological oxygen demand (BOD_1), total alkalinity (TA), total hardness (TH), ortho-phosphorus (Ortho-P); ammonia-nitrogen (NH_3-N), nitrite-nitrogen (NO_2-N), nitrate-nitrogen (NO_3-N) and total available nitrogen (TAN) of water (Wetzel and Likens, 1991; APHA, 1995). The pH, organic carbon (SOC), available nitrogen (SAN) and available phosphorus (SAP) of soil samples were determined following the methods described by Jackson (1959).

2.5.2 Proximate composition of feed, bioflocand shrimp

Proximate analyses (on dry matter basis) of experimental feed, biofloc, shrimp (Tables 2–4) were performed as per the standard method of AOAC (2005). Dried biofloc samples were ground and processed for proximate analysis. The level of moisture was determined in hot air oven at 105 °C temperature for overnight until constant weight was obtained. Crude protein (CP) and ether extract (EE) were estimated using

micro-Kjeldahl method (KEL PLUS, Pelican Equipments, India) and Soxhlet extraction method (SOCS PLUS, Pelican Equipments, India), respectively. Crude fiber (CF) (fat free samples) was estimated by using FIBRA PLUS (Pelican Equipments, India). The dried feed and whole fish sample were subjected to 550 °C for 5 hours in a Muffle furnace (Macro Scientific, India) to measure the total ash (TA) content. The nitrogen free extract (NFE) of the diets and total carbohydrate (TC) of carcass were calculated using following formulae: NFE (%) = [100–(CP% + EE% + TA% + CF%)] and TC= [100-(CP% + EE% + TA%)]. Digestible energy (DE) of the diets was calculated as per the following formula given by Halver (1976): DE (kcal/100 g) = [(CP% × 4) + (EE% × 9) + (NFE% × 4)]. The protein (P) to energy (E) ratio was calculated by using following formula: P: E (mg CP / kcal DE) = [(CP% × 1000)/DE].

Table 2. Proximate composition of commercial feed used for *L. vannamei*.

Proximate composition	(% dry matter basis)
Moisture (%)	7.2
Crude protein (%)	42.02
Ether extract (%)	6.64
Crude fibre (%)	7.32
Total ash (%)	4.54
Nitrogen free extract (%)	39.48
DE (kcal/100 g)	385.76
P : E (mg protein / kcal DE)	108.93

2.5.3 Microbial population

Enumeration of aerobic heterotrophic bacteria was done by following standard method (APHA, 1995). Different types of mineralizing bacterial populations were enumerated viz. nitrogen fixing bacteria (NFB), ammonifying bacteria (AB), ammonia oxidizing bacteria (AOB), denitrifying bacteria (DNB) (Alexander, 1978), phosphate solubilizing bacteria (PSB) (Rao, 1977) and cellulose decomposing bacteria (CDB) (Gupta et al., 2012). Enumeration and isolation of bioluminescent and probiotic bacterial populations were followed according to Kannahi and Sivasankari (2014) and Anand et al. (2014), respectively. Luminous bacterial (LB) populations were identified as per the taxonomic scheme of Alsina and Blanch (1994) and Abraham et al. (1999).

2.5.4 Collection and analysis of plankton

A conical plankton net made up of the bolting silk cloth (No. 21 with 77 meshes per square cm.) was used to collect the plankton samples. About 50 liters of water from each pond was collected from randomly selected locations with the help of 1000 ml beaker and pooled together for filtering, 20 ml filtrate

Table 3. Growth performance of *L. vannamei* in different pond treatments (mean ± standard error; $n = 3$).

Parameters	B	NB	F	C
Survival percentage	87.89^{c}	87.76^{c}	81.52^{b}	78.66^{a}
Initial weight (mg)	15.50 ± 0.25^{a}	15.50 ± 0.25^{a}	15.50 ± 0.25^{a}	15.50 ± 0.25^{a}
Final body weight (g)	32.57 ± 0.03^{c}	33.82 ± 0.01^{d}	24.22 ± 0.01^{b}	14.27 ± 0.01^{a}
Body weight gain (g)	16.98 ± 4.27^{c}	17.59 ± 4.46^{c}	12.40 ± 3.20^{b}	5.85 ± 1.80^{a}
SGR	30.99 ± 3.56^{c}	32.18 ± 3.22^{c}	23.04 ± 2.95^{b}	13.57 ± 2.15^{a}
Feed intake (g/shrimp)	51.34^{b}	52.98^{b}	37.02^{a}	–
FCR	1.58 ± 0.35^{a}	1.57 ± 0.31^{a}	1.71 ± 0.29^{b}	–
FCE	76.05 ± 13.43^{a}	75.00 ± 11.45^{a}	72.86 ± 13.82^{b}	–
PER	1.83 ± 0.64	1.78 ± 0.58	1.81 ± 0.52	–
ANPU	42.81^{c}	31.42^{b}	25.03^{a}	–

[a, b, c] Means superscripted with different letters in rows are significantly different ($P < 0.05$).

Table 4. Proximate composition (% dry matter basis) of biofloc in different pond treatments (mean ± standard error; $n = 3$).

Parameter (%)	B	NB
Moisture	51.37 ± 0.09^{a}	53.18 ± 0.05^{b}
Crude protein	19.63 ± 1.03^{b}	18.75 ± 0.74^{a}
Ether extract	0.56 ± 0.02^{b}	0.53 ± 0.02^{a}
Carbohydrate	23.02 ± 0.72^{b}	22.00 ± 0.67^{a}
Ash	5.39 ± 1.57	5.52 ± 1.01
NFE	51.4	53.2
DE (kcal 100 g^{-1})	289.16	292.57
P: E (mg protein. kcal DE^{-1})	6.78	6.41

[a, b, c] Means superscripted with different letters in rows are significantly different ($P < 0.05$).

was preserved in 4% formalin solution and stored in labeled vials for subsequent quantitative and qualitative analyses under a binocular microscope. The samples were analyzed by drop count method (APHA, 1995) for enumeration of phytoplankton populations. One drop of sample was pipetted out by dropper on a glass slide and covered with cover slip. Finally, the phytoplankton organisms were counted and calculated though the formula:

$$\text{No. of phytoplankton/drop (A)} = \frac{\text{Area of coverslip} \times \text{total individuals counted in all fields}}{\text{Area of one field} \times \text{no. of fields observed}}$$

Area of cover slip = 1521.1429 mm^2; area of one field = 0.0805 mm^2; no. of phytoplankton / ml of concentrate = A×20 = b (1ml = 20 drops)

$$\text{No. of phytoplankton/liter of water} = \frac{(\text{b} \times \text{ml of concentrate} \times 1000\,(\text{ml})}{\text{Volume of water sample (ml)}}$$

Whereas, zooplankton samples were counted and calculated following the Sedgwick Rafter Cell (SRC) method (APHA 2005) through the formula:

$$\text{No. of zooplankton/ml (N)} = [\text{C} \times \text{Area of SRC}\,(1000\,mm^2)]/\text{A} \times \text{D} \times \text{E}$$

C = Number of organisms recorded per 10 fields; A = Area of one microscope field (1.369 mm^2); D = Depth of field (S.R.C. depth) in mm (1 mm); E = Number of fields counted; Number of zooplankton / liter = [N × Volume of concentrate (ml)] / Volume of water filtered (L).

2.5.5 Survival rate, growth response and feeding efficiency of *L. vannamei*

The survival rate of *L. vannamei* was calculated at the end of experiment wascalculated from the number of shrimps at harvestin the respective treatments. Growth response in terms of body weight was recorded at 15 days intervals from each treatment. Specific growth rate (SGR), feed conversion ratio (FCR), feed conversion efficiency (FCE%), protein efficiency ratio (PER) and apparent net protein utilization (ANPU) was calculated as follows:

Body weight gain (g) = Final body weight (g) − Initial body weight (g)

SGR (%) = [(*ln* final wet body weight) − (*ln* initial wet body weight)/Number of days] × 100

Feed intake (g/fish) = [Total feed intake (g) / Total number of fish/shrimp]

FCR = [Total dry feed fed (dry weight in g) / Body weight gain (wet weight in g)]

FCE (%) = (Body weight gain (wet weight in g) × 100)/ Total dry feed fed (dry weight in g)

PER = [Live body weight gain (wet weight in g) / Crude protein fed (dry weight in g)]

ANPU (%) = [100 × (Final carcass protein-Initial carcass protein) / Protein fed (dry weight in g)]

2.6 Economic analyses

The economic analysis of production performance of *L. vannamei* in biofloc system was carried out based on the results obtained in the present study by using the following parameters:

Final total population numbers of shrimp (nos.) = Initial stocking number in 1000 m3 pond × Survival rate (%)

Final total biomass of shrimp (g) = Survival rate (%) × Final total population numbers of shrimp (nos.)

$$\text{Total feed offered (kg)} = \frac{\text{Final total biomass of shrimp (g)} \times \text{Feed offered per shrimp (g)}}{1000}$$

Feed cost per kg shrimp (₹) = [Total feed offered (kg) / Final total biomass of shrimp (kg)] × Cost of 1 kg. feed.

2.7 Statistical analyses

Once the assumptions of normality and homogeneity ofvariances were convened (Sokal and Rohlf, 1995), one way analysis of variance (Montgomery, 2001) was applied to test the significance in difference among the treatments followed by Duncan's multiple range test (DMRT) to findout the homogeneity among the mean of every possible pair of treatment combinations (Microsoft Excel 2007 suite). The results were accepted at 5% level of significance ($P < 0.05$). Correlation co-efficient (r) was applied to establish relationship between selective parameters.

3 Results

3.1 Survival and growth

Survival rate of *L. vannamei* was increased ($P < 0.05$, ANOVA) in both the biofloc treatments (B and NB) compared to other treatments (F and C) with significant difference among the treatments. Shrimp growth in terms of live weight gradually increased in all the treatments with highly significant ($P < 0.05$; ANOVA) difference among them. The highest mean live weightvalue (17.59 g) was achieved in NB which was 3.61%, 41.85% and 2.0 times more than B (16.98 g), F (12.40 g) and C (5.85 g) respectively. Specific growth rate was also highest in NB followed by B, F, and C (Tab. 3).

3.2 Feeding efficiency

The overall highest mean of FCR was estimated in B and lowest in F (Tab. 3). Though overall treatment difference remained significant ($P < 0.05$; ANOVA), difference between B and NB remained insignificant (DMRT). FCE (%) was highest in B and lowest in NB (Tab. 3).

3.3 Proximate composition of biofloc and shrimp

There was significant difference ($P < 0.05$) in proximate composition of biofloc in B and NB (Tab. 4). Overall mean value of biofloc volume was marginally higher (15.33 ml l^{-1})

Table 5. Proximatefs composition (% dry matter basis) of *L. vannamei* cultured in different pond treatments (mean ± standard error; $n = 3$).

Parameters (%)	B	NB	F	C
Moisture	75.24 ± 0.81^{a}	76.19 ± 0.48^{a}	76.76 ± 0.72^{a}	77.35 ± 0.49^{a}
Crude protein	24.58 ± 2.14^{a}	26.29 ± 2.27^{b}	$22.54 \pm 2{,}23^{c}$	17.33 ± 1.17^{d}
Ether extract	6.24 ± 0.50^{a}	6.72 ± 0.59^{b}	5.80 ± 0.41^{a}	4.03 ± 0.22^{d}
Ash	2.91 ± 0.09^{a}	2.97 ± 0.12^{a}	2.92 ± 0.07^{a}	2.63 ± 0.16^{b}
Crude fiber	0.98 ± 0.11^{a}	1.01 ± 0.12^{a}	1.02 ± 0.16^{a}	1.04 ± 0.24^{a}
Total carbohydrate	66.26 ± 2.1^{a}	64.01 ± 1.86^{a}	68.74 ± 2.12^{c}	76.00 ± 2.18^{d}

[a, b, c, d] Means superscripted with different letters in each row are significantly different ($P < 0.05$).

Table 6. Physico-chemical, biological and microbial parameters of water and soil in different pond treatments (mean ± standard error; $n = 3$).

Parameters	Treatments			
Water quality	B	NB	F	C
Temperature (°C)	35.50 ± 1.50	35.50 ± 1.50	35.50 ± 1.50	35.50 ± 1.50
pH in water	7.88–8.45	7.53–8.32	7.51–8.42	7.56–8.42
TDS (mg l^{-1})	140.83 ± 15.02^{b}	134.17 ± 12.44^{b}	111.25 ± 8.73^{a}	110.83 ± 8.86^{a}
DO (mg l^{-1})	10.26 ± 0.60^{d}	9.50 ± 0.38^{c}	9.16 ± 0.36^{b}	8.73 ± 0.25^{a}
BOD_1 (mg l^{-1})	4.81 ± 0.17^{a}	4.70 ± 0.16^{a}	4.99 ± 0.07^{b}	4.92 ± 0.06^{b}
Alkalinity (mg l^{-1})	202.96 ± 10.01^{c}	108.13 ± 5.24^{a}	161.58 ± 3.15^{b}	157.92 ± 2.87^{b}
Hardness (mg l^{-1})	550.83 ± 15.63^{c}	497.42 ± 11.52^{a}	528.75 ± 6.00^{b}	521.08 ± 8.21^{b}
Ortho-P (mg l^{-1})	0.087 ± 0.00^{c}	0.078 ± 0.00^{b}	0.075 ± 0.00^{b}	0.068 ± 0.00^{a}
NH_3-N (mg l^{-1})	0.368 ± 0.02^{b}	0.446 ± 0.03^{d}	0.392 ± 0.01^{c}	0.346 ± 0.00^{a}
NO_2-N (mg l^{-1})	0.004 ± 0.00^{a}	0.003 ± 0.00^{a}	0.006 ± 0.00^{c}	0.004 ± 0.00^{b}
NO_3-N (mg l^{-1})	0.35 ± 0.04^{c}	0.45 ± 0.05^{d}	0.25 ± 0.02^{b}	0.18 ± 0.01^{a}
TAN (mg l^{-1})	0.72 ± 0.05^{c}	0.90 ± 0.07^{d}	0.65 ± 0.03^{b}	0.53 ± 0.00^{a}
TAN:P ratio	8.25 ± 0.30^{b}	11.53 ± 0.70^{c}	8.67 ± 0.21^{b}	7.87 ± 0.19^{a}
Total phytoplankton (nos. × 1000 l^{-1})	9.07 ± 0.18^{a}	7.35 ± 0.22^{b}	6.42 ± 0.27^{c}	4.39 ± 0.17^{d}
Total zooplankton (nos. × 1000 l^{-1})	2.52 ± 0.14^{a}	2.13 ± 0.18^{b}	1.86 ± 0.15^{c}	1.41 ± 0.16^{d}
Soil quality				
Soil pH	7.81–8.42	7.71–8.41	7.71–8.41	7.81–8.39
SOC (mg g^{-1})	2.39 ± 0.32^{c}	2.20 ± 0.26^{b}	2.08 ± 0.29^{b}	1.53 ± 0.18^{a}
SAP (mg kg^{-1})	59.07 ± 5.15^{c}	54.82 ± 4.97^{b}	50.63 ± 4.54^{b}	40.34 ± 1.60^{a}
SAN (mg kg^{-1})	590.02 ± 44.52^{c}	664.69 ± 51.46^{d}	555.80 ± 40.33^{b}	423.58 ± 18.46^{a}
N: P ratio	10.15 ± 0.33^{a}	12.32 ± 0.45^{d}	11.25 ± 0.58^{c}	10.53 ± 0.24^{b}

[a, b, c, d] Means superscripted with different letters in rows are significantly different ($P < 0.05$).
The statistical analysis was done separately for water and soil.

in B than NB (15.22 ml l^{-1}). Proximate analyses of biofloc showed that crude protein and carbohydrate content was significantly higher in B compared to NB with insignificant difference between the treatments with regards to ether extract and ash content. As a result, digestible energy was higher in NB than B but P: E was superior in the later. However, moisture content and NFE (%) were significantly higher in NB (Tab. 4).

Proximate composition of cultured shrimp species showed significantly higher values of crude protein and ether extract in NB compared to B. However, carbohydrate content was highest in C (Tab. 5).

3.4 Water and soil quality parameters

All the water quality parameters are presented in Table 6. Surface water temperature varied between 30 and 38 °C with insignificant variation among all the treatments. pH of water remained alkaline (7.51–8.45) in all the treatments. TDS, DO, alkalinity, hardness and ortho-P were significantly ($P < 0.05$; ANOVA) higher in B compared to NB, but inorganic nitrogen like NH_3-N, NO_3-N, TAN and TAN: P ratio were higher in the later (Tab. 6). Soil pH remained alkaline in all the treatments. Though, available-P of soil was significantly higher in B, available-N and N: P ratio were significantly higher in NB.

3.5 Plankton

Total phytoplankton and zooplankton population differed significantly ($P < 0.05$; ANOVA) among the treatments. Total phytoplankton populations were highest in B (9068 nos. l^{-1}) followed by NB (7348.61 nos. l^{-1}), F (6418.19 nos. l^{-1}) and C (4389.58 nos. l^{-1}). Likewise, total zooplankton populations

Table 7. Microbial populations in water and soil in *L. vannamei* culture (mean ± standard error; $n = 3$).

Parameters (CFU × 104 ml^{-1})	Water				Soil			
	B	NB	F	C	B	NB	F	C
AHB	6.00 ± 0.76^{d}	5.70 ± 0.76^{c}	4.18 ± 1.60^{b}	3.48 ± 1.33^{a}	10.53 ± 1.16^{d}	9.29 ± 0.91^{c}	8.50 ± 0.79^{b}	7.81 ± 0.69^{a}
NFB	3.61 ± 0.60^{c}	4.83 ± 0.79^{d}	3.05 ± 0.39^{b}	2.63 ± 0.27^{a}	8.94 ± 0.88^{c}	9.77 ± 0.97^{d}	7.29 ± 0.63^{b}	5.66 ± 0.22^{a}
AB	5.53 ± 0.81^{c}	5.36 ± 0.72^{c}	3.37 ± 0.53^{b}	2.11 ± 0.16^{a}	11.00 ± 1.08^{d}	10.33 ± 0.97^{c}	8.32 ± 0.81^{b}	6.52 ± 0.42^{a}
AOB	4.93 ± 0.81^{c}	3.48 ± 0.64^{b}	1.84 ± 0.12^{a}	1.60 ± 0.07^{a}	12.04 ± 0.83^{d}	11.43 ± 0.77^{c}	8.27 ± 0.19^{b}	7.79 ± 0.11^{a}
DNB	5.63 ± 0.83^{d}	4.32 ± 0.70^{c}	3.43 ± 0.52^{b}	2.61 ± 0.28^{a}	9.20 ± 0.91^{c}	8.62 ± 0.83^{b}	5.50 ± 0.39^{a}	5.02 ± 0.27^{a}
CDB	5.35 ± 0.79^{d}	4.78 ± 0.68^{c}	3.75 ± 0.48^{b}	2.90 ± 0.22^{a}	9.86 ± 0.81^{d}	8.94 ± 0.65^{c}	7.58 ± 0.49^{b}	6.13 ± 0.27^{a}
PSB	2.97 ± 0.46^{c}	2.33 ± 0.33^{b}	2.14 ± 0.32^{b}	1.83 ± 0.26^{a}	7.42 ± 0.52^{d}	6.50 ± 0.41^{c}	5.26 ± 0.24^{b}	4.92 ± 0.15^{a}
LB	0.64 ± 0.14^{b}	0.58 ± 0.14^{a}	1.77 ± 0.08^{d}	1.30 ± 0.04^{c}	2.32 ± 0.17^{b}	1.90 ± 0.18^{a}	3.24 ± 0.07^{c}	3.06 ± 0.07^{c}
Bacillus sp.	1.28 ± 0.19^{d}	0.78 ± 0.10^{c}	0.53 ± 0.04^{b}	0.35 ± 0.04^{a}	4.00 ± 0.30^{c}	2.84 ± 0.18^{b}	2.44 ± 0.12^{b}	1.98 ± 0.07^{a}
Lactobacillus sp.	3.00 ± 0.22^{d}	1.93 ± 0.17^{c}	1.77 ± 0.07^{b}	1.50 ± 0.11^{a}	4.96 ± 0.29^{c}	3.57 ± 0.21^{b}	3.19 ± 0.03^{b}	2.75 ± 0.11^{a}

[a, b, c, d] Means superscripted with different letters in each row are significantly different ($P < 0.05$).
The statistical analysis was done separately for water and soil.

were also highest in B (2526.35 nos. l^{-1}) followed by NB (2134.56 nos. l^{-1}), F (1861.53 nos. l^{-1}) and C (1406.15 nos. l^{-1}) (Tab. 6).

3.6 Microbial populations

Aerobic heterotrophic bacterial populationin both water and soil differed significantly ($P < 0.05$; ANOVA) among the treatments as the highest value was encountered in B (6−10.53 CFU × 10^4 ml^{-1}) which was 5.43−13.32% higher than NB (Tab. 7). Likewise, highest mean population of NFB was recorded in NB, whereas, significantly ($P < 0.05$; ANOVA) high microbial population load of AOB, AB, DNB, CDB and PSB were encountered in B. Significant differences were also observed for luminous *Vibrio* and probiotic (*Bacillus* sp. and *Lactobacillus* sp.) populations in all the treatments (Tab. 7). Luminous *Vibrio* population recorded in F was 36.22−208% more than B and NB. The highest mean population of *Bacillus* sp. and *Lactobacillus* sp. were observed in B which was 40.91−267% and 39.14−100% higher respectively, than other treatments employed.

3.7 Economic benefits of biofloc based culture

As the price of pellet feed at the time of experimentation was ₹ 62.00 kg^{-1}, total feed cost towards production of shrimp was highest in F (₹ 106.14 kg^{-1} shrimp) followed by B (₹ 98.19 kg^{-1} shrimp) and NB (₹ 97.58 kg^{-1} shrimp). Therefore, feed cost was reduced both in B (8.10%) and NB (8.77%) compared to conventionalfeedbased culture system (F).

4 Discussion

Survivability of shrimp was higher (>87%) in biofloc treatments in which high C:N ratio induced the biofloc microbial diversity and nutritional quality that in turn enhanced the survival rate of *L. vannamei*. Such observations fit with the earlier study of Panigrahi et al. (2019). The superiority of biofloc technology over feed supplemented traditionalproduction system has been well established in the present study as live weight gain of *L. vannamei* was 62.04–67.89% more in the biofloc applied systems. Moreover, both the biofloc media (B and NB) were proved to be highly efficient in reducing the FCR value by 7.60% and 8.18% respectively as compared to the conventional feeding (F). Therefore, the media used to in situ development of biofloc was quite economical in replacing costly compound diet to the cultured shrimp. This was in conformity with the earlier findings of Megahed (2010).

Biofloc not only reduced the feed cost but also improved the whole production status of the system both without neem (B) and neem (NB) based system which resulted in more growth of *L. vannamei*. Application of carbon rich biofloc media directly influenced the total aerobic heterotrophic bacterial population in the culture system. The additional carbon acted as readily available energy source to the microbes. The enhanced carbon pool ameliorated the C:N ratio which favoured heterotrophic production. The result of this study established that heterotrophic flora was more pronounced in biofloc system than the conventional culture process of *L. vannamei* with feed alone. Earlier studies (Avnimelech, 1999; Shan and Obbard, 2001) also reported that addition of carbon source enhances bacterial abundance inpond culture system of fish andshellfishes.

The greater abundance of phyto and zooplankton population along with primary productivity was also favoured by both the media used for biofloc production indicated that biofloc not only supported the stock in providing valuable single cell microbial protein but also secondarily enriched the productivity of the whole system by triggering the planktonic production (Azim et al., 2002). This was evidenced by the positive relationship between primary productivity and growth of shrimp in both B ($y = -0.000x^2 + 0.035x + 0.801$; $R^2 = 0.611$) and NB ($y = -0.000x^2 + 0.04x + 0.729$; $R^2 = 0.743$). Moreover, higher concentration of dissolved oxygen coupled with relatively lower values of BOD_1 in either of the biofloc

Table 8. Co-relation between nutrient parameters and mineralizing bacterial populations in water and soilin *L. vannamei* culture.

Parameters	B		NB	
	Water	Soil	Water	Soil
NH_3-N Vs AB	$R^2 = 0.462$	–	$R^2 = 0.605$	–
NH_3-N Vs NFB	$R^2 = 0.901$	–	$R^2 = 0.716$	–
NH_3-N Vs AOB	$R^2 = 0.562$	–	$R^2 = 0.887$	–
NH_3-N Vs DNB	$R^2 = 0.530$	–	$R^2 = 0.902$	–
NO_3-N Vs AB	$R^2 = 0.880$	$R^2 = 0.877$	$R^2 = 0.917$	$R^2 = 0.952$
NO_3-N Vs AOB	$R^2 = 0.782$	$R^2 = 0.948$	$R^2 = 0.631$	$R^2 = 0.900$
NO_3-N Vs NFB	$R^2 = 0.439$	$R^2 = 0.911$	$R^2 = 0.890$	$R^2 = 0.953$
NO_3-N Vs DNB	$R^2 = 0.833$	$R^2 = 0.949$	$R^2 = 0.724$	$R^2 = 0.877$
Ortho-P Vs PSB	$R^2 = 0.791$	$R^2 = 0.732$	$R^2 = 0.507$	$R^2 = 0.790$
SAP Vs PSB	$R^2 = 0.682$	$R^2 = 0.936$	$R^2 = 0.665$	$R^2 = 0.782$

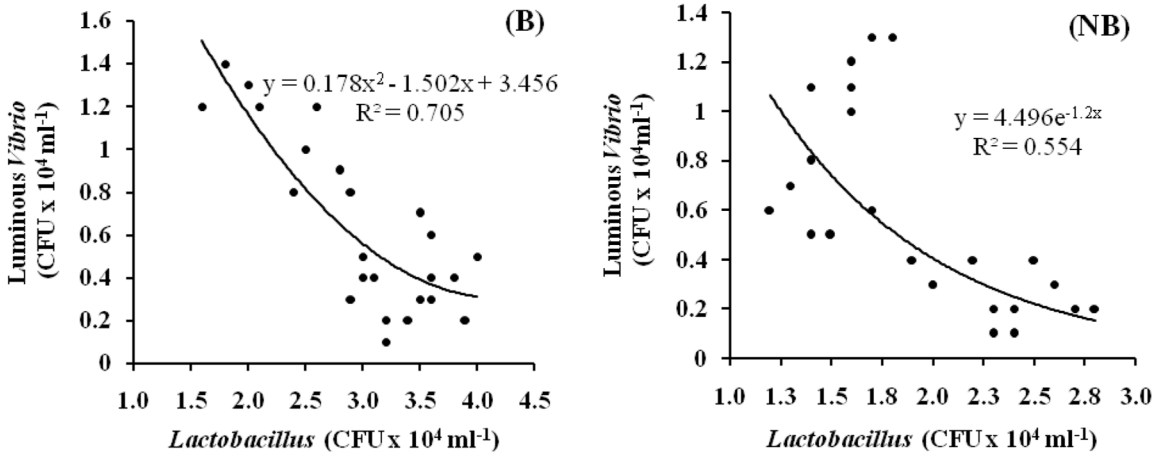

Fig. 1. Fitted relationship between *Lactobacillus* sp. and luminous *Vibrio* sp. in water in without (B) and with neem (NB) based biofloc culture system of *L. vannamei*.

treatments proved it's superiority over the conventional feed based intensive or semi-intensive culture system of *L. vannamei*. This might be due to the higher abundance of nutrient cycling microbes in the biofloc enriched media which has resulted in more supply of N and P to the primary producers operating in the base level of a production system. This was manifested in the fitted relationship between different nutrient parameters of both water and soil with biogeochemical cycling microbes operating in both the biofloc media applied (Tab. 8).

Although, the population density of *Bacillus* sp. and *Lactobacillus* sp. significantly increased in both the media, biofloc with neem exerted negative impact upon them as evidenced from the significantly lower population size compared to the medium without neem. Antimicrobial compounds viz. azadirachtin from the neem leaves might have impacted negatively upon the microbes (Harikrishnan et al., 2003; Dhayanithi et al., 2010). Gupta et al. (2016) reported that aqueous extract of neem leaves hasshown bactericidal activity against *Lactobacillus acidophilus* and *L. rhamnosus*. Conspicuously, such impact was not pronounced in either presumptive *Vibrio* or luminous bacteria as well where the population size declined through out under both the media applied. Moreover, the bacillus community particularly *Lactobacillus* sp. negatively impacted the luminous bacterial population. As a result, the cumulative increase of bacillus population with a concomitant decrease of luminous bacteria of water in either of the media strongly established polynomial relationship with a high degree of fit (Figs. 1–3). This implied that luminous bacterial population controlling mechanism by *Bacillus* sp. and *Lactobacillus* sp. was highly pronounced in either of the biofloc media. Earlier, Pattukumar et al. (2010) stated that the load of *Vibrio parahaemolyticus* decreased with simultaneous increase of probiotic (*Bacillus* sp.) through application of probiotic in *Penaeus monodon* culture pond. Increase in total dissolved solids (For B: $y = 4E{-}05x^2 - 0.019x + 2.455$; $R^2 = 0.778$) and for NB: $y = 3E{-}05x^2 - 0.018x + 2.407$; $R^2 = 0.827$) and salinity ((For B: $y = 5.565e^{-0.13}x$; $R^2 = 0.786$ and for NB: $y = 5.444e^{-0.13x}$; $R^2 = 0.745$) of water negatively impacted the luminous bacterial population in both and played synergistically with *Bacillus* sp. community in reducing the former.

Though biofloc volume did not differ between B and NB, significant difference in the protein content between them

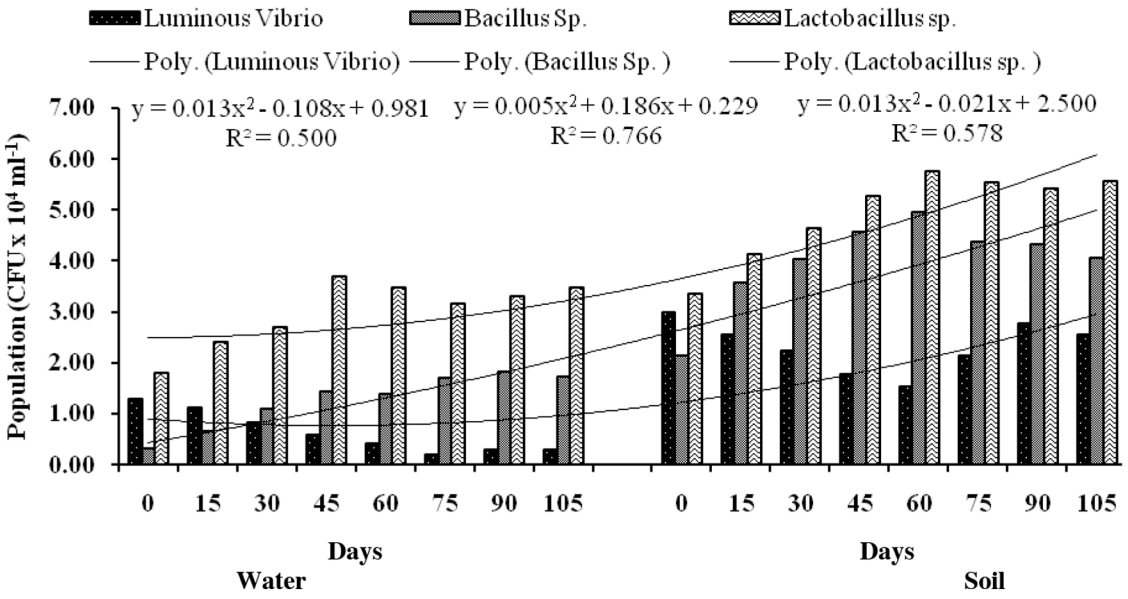

Fig. 2. Fitted relationship of cumulative changes in population of luminous *Vibrio* sp., *Bacillus cereus*, *Lactobacillus* sp. in without neem based biofloc culture system of *L. vannamei*.

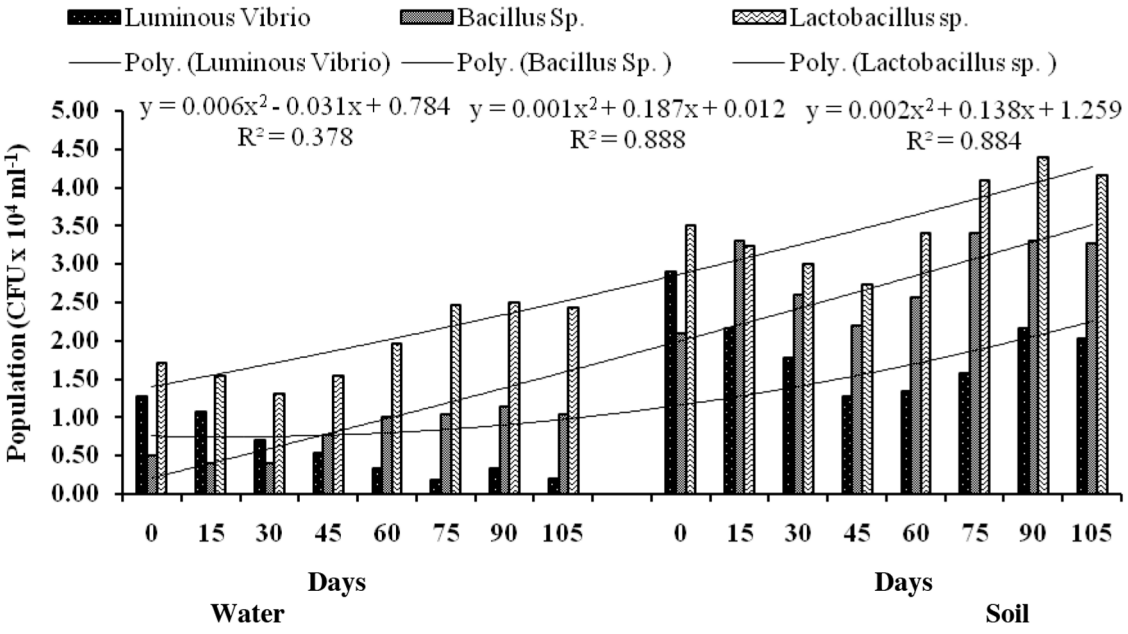

Fig. 3. Fitted relationship of cumulative changes in population of luminous *Vibrio* sp., *Bacillus cereus*, *Lactobacillus* sp. in with neem based biofloc culture system of *L. vannamei*.

indicated superiority of the former in terms of protein and lipid content that was vital nutrients in shrimp feeding. Rice based biofloc medium (B) might have favoured the C:N ratio in providing readily available carbon source as starch. Moreover, in absence of such source, boiled neem leaves in (NB) added more nitrogen into the media as reflected in the results of the inorganic nitrogen concentration of water thereby widening the C:N ratio. Also, such nitrogen enrichment in NB results in more N:P ratio of both water and soil compared to B. This was discernible from the fitted relationship of either NH_3-N or NO_3-N and N:P ratio of water in which such relationship was more pronounced in NB ($R^2 = 0.63-0.82$) compared to B ($R^2 = 0.48-0.76$) (Figs. 4 and 5).

The proximate composition of shrimp was dependent on the proximate composition of natural food from plankton, feed and biofloc. The higher crude protein was observed in both the biofloc treatments ranged from 8.31−29.49% (B) and 14.26−34.07% (NB) than F and C. Nutritional qualities of shrimp increased in both the biofloc system compared to without biofloc system which confirmed that high proximate compo-

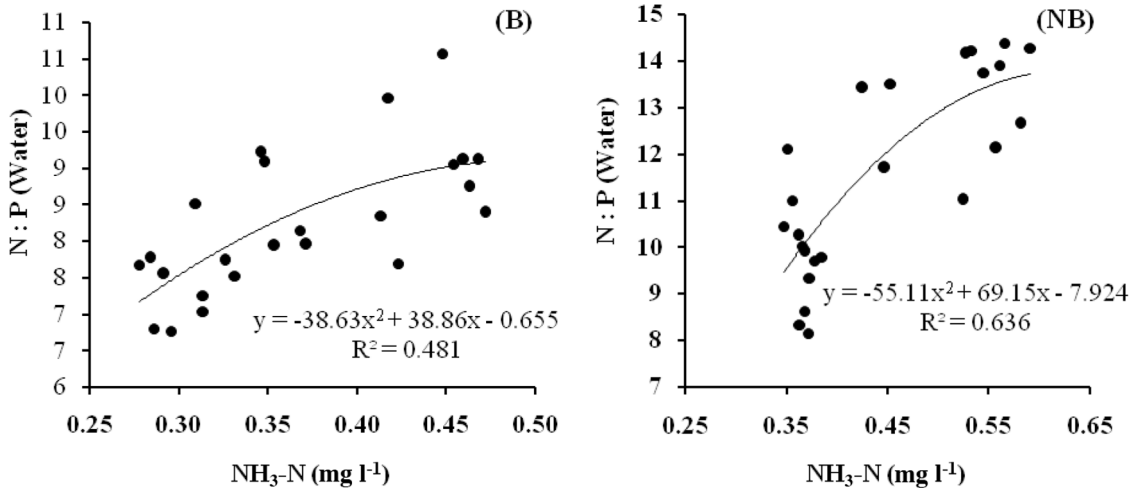

Fig. 4. Fitted relationship between NH_3-N and N:P ratio in without neem (B) and neem based (NB) biofloc system.

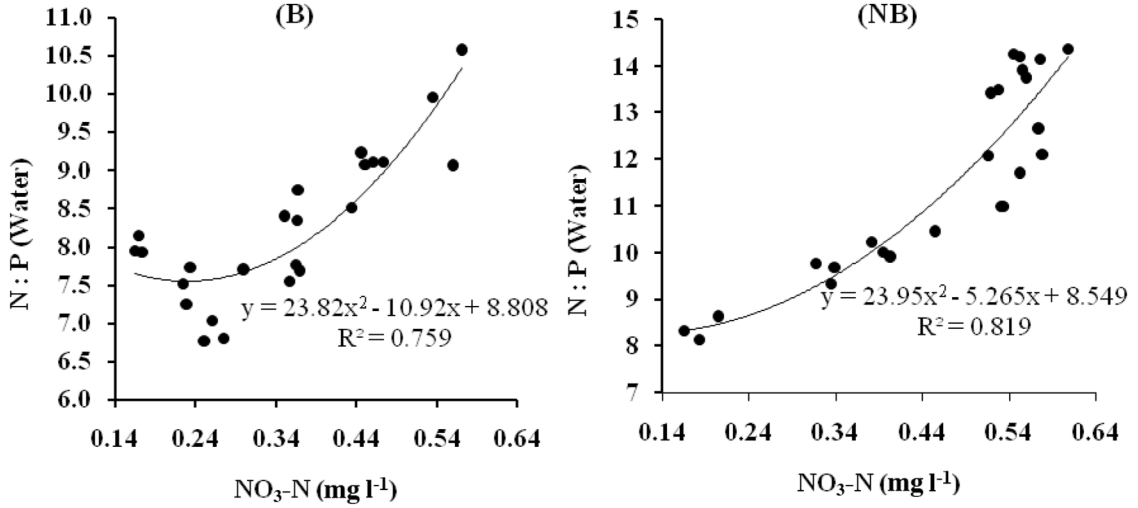

Fig. 5. Fitted relationship between NO_3-N and N:P ratio in without neem (a) and neem based (b) biofloc system.

sitions of biofloc led to an improved growth rate of shrimp by 26.99−65.52% in B and 29.53−66.72% in NB than F and C. The findings were similar to Rajkumar et al. (2016). The proximate composition of biofloc revealed that the level of nutritional components enhanced shrimp growth (Cuzon et al., 2004).

5 Conclusion

The overall improvement of the ecological health of the culture systems under biofloc technology was evident in the present study concerning water and soil quality parameters, as well as pathogenic *Vibrio* load of the culture systems. Moreover, biofloc with neem supplementation has been proved beneficial in controlling disease-causing luminous *Vibrio* population in *L. vannamei* culture probably through their antimicrobial effect. It is suggested that neem leaves can be used in low dose (<2 kg l^{-1}) in composing the biofloc medium for application in pond culture system of *L. vannamei*, which may increase the profit level of the shrimp farmers through the enhancement of survival and growth of shrimp.

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