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Carbon: Nitrogen (C:N) ratio level variation influences microbial community of the system and growth as well as immunity of shrimp (*Litopenaeus vannamei*) in biofloc based culture system

A. Panigrahi[a,*], C. Saranya[a], M. Sundaram[a], S.R. Vinoth Kannan[a], Rasmi R. Das[a], R. Satish Kumar[b], P. Rajesh[a], S.K. Otta[a]

[a] *Crustacean Culture Division, ICAR-Central Institute of Brackishwater Aquaculture, #75, Santhome High Road, Raja Annamalai Puram, Chennai, Tamil Nadu 600028, India*

[b] *School of Chemical Engineering, Yeungnam University, 280 Daehak-Ro, Gyeongsan, Gyeongbuk, 38541, South Korea*

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A B S T R A C T

Biofloc technology (BFT) is a novel modern aquaculture farming technique used to reduce toxic nitrogen concentration, act as *in situ* food source and eradicate pollutants using carbon and therefore to control C:N ratio in an aquaculture system. In this study, effect of different C:N ratios of a biofloc based system on water quality such as the level of Total ammonia nitrogen (TAN) nitrite-nitrogen (NO_2^-–N) and nitrate nitrogen (NO_3^-–N) were explored. Further, the growth and immunity status of shrimp *L. vannamei* under the influence of different C:N ratios were evaluated. Two of the C:N ratios (15 and 20) could significantly ($P < 0.05$) reduce TAN, NO_2-N and NO_3-N levels (0.456 ± 0.01, 0.145 ± 0.09, and 0.102 ± 0.02 ppm) compared to control (1.45 ± 0.1, 0.749 ± 0.14 and 0.675 ± 0.16 ppm). Large variations in the frequency distribution of operational taxonomic units (OTUs) for the bacterial community in water with different C:N ration (BFT) and control were observed. Vibrios often considered as opportunistic pathogens, where the most dominant bacterial flora of water in control (79%) and C:N5 (37%) group. In C:N10, *Thauera* (62%) was most represented genus. Similarly, *Attheyaceae* (56%), followed by *Peridiniaceae* (30%) were the most dominant groups in C:N15 treatment. The diversity of bacterial flora was more spread in C:N20 treatments with *Psychrobacter* (26%), *Proteobacteria* (25%) and *Peridiniaceae* (20%) as the major groups. The trend of Vibrio dominance decreased with the increase in C:N ratios and thus confirming the dominance of heterotrophic bacteria in high C:N ratio groups. Upon challenge with pathogens, shrimps from C:N10, C:N15 and C:N20 groups showed significantly higher survival ($P < 0.05$) compared to the C:N5 and control group. Similarly, better growth rate was also observed in BFT tanks compared to control both during the culture and at harvest. Comparatively higher expression of four immune-related genes (ras-related nuclear gene (RAN), serine proteinase gene (SP), prophenoloxidase activating enzyme (PPAE), and crustin were observed in different C:N ratio ponds than control and these were in increasing trend with the C:N ratio. Gene expression analysis showed that the transcripts of those immune genes were significantly increased among all C:N treatments than that of control. Overall, these findings demonstrated that with optimum C:N ratio, BFT can be used to optimize the bacterial community composition for both optimal water quality and optimal shrimp health. This study thus indicates the possibility of obtaining better performance of *L. vannamei* culture with proper adjustment of C:N ratio in a biofloc based system.

1. Introduction

Extensive use of inputs through intensification of shrimp aquaculture practice brings stress and makes the animals susceptible to different diseases. Generation of toxic metabolites such as ammonium and nitrite from the accumulated aquaculture wastes in the form of feces and unutilized feed is main culprit behind this stress. It results in severe adverse implication on the overall production bringing huge economic loss to farmers [1–6]. As intensification is inevitable to achieve high production, modification of existing culture practices has

* Corresponding author. Crustacean Culture Division, ICAR-Central institute of Brackishwater Aquaculture, #75, Santhome High Road, Raja Annamalai Puram, Chennai, Tamil Nadu, 600028, India.

E-mail addresses: apanigrahi2k@gmail.com, panigrahi@ciba.res.in (A. Panigrahi).

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become quite necessary for the establishment of better environment; to maintain good health and growth of animals.

Biofloc based culture practice is one such technology (BFT) during recent time which has added several advantages including of biosecure system due to zero or minimal water exchange, improved water quality through the self-generated bioremediation process and improved growth and immune system of shrimps through diverse heterotrophic bacterial system. In addition to this, biofloc also serves as a high nutrients food source rich in amino acids, proteins, fatty acids and lipids in the form of different microorganisms, and thus substantially reduces external feed supply to make it more economical. The diverse microbial community of flocs not only provides supplemental nutrition, but also acts as consumers of dissolved oxygen, as nutrient recyclers, and as a food source for different microbial diversity from higher trophic levels in aquaculture [1,5,7–9]. Several studies reported that the composition, structure, and stability of bio-flocs could be affected by diverse types of organic carbon sources (molasses, corn, wheat, glucose acetate, glycerol, and tapioca) and their ratio. Different carbon-nitrogen ratio also makes the differences in the bioflocs nutrients composition such as carbohydrate, protein, lipid and fatty acid [9–14].

In most of the aquaculture feeds, the C: N ratio is about 7–10:1, whereas bacterial population present in ponds needs around 20 units of carbon to assimilate one unit of nitrogen [15]. The heterotrophic bacteria populations in aquaculture ponds will not get expanded to desired level with such a low C:N ratio. Therefore, addition of extra carbon sources becomes inevitable to increase the heterotrophic bacterial population to a dense mass in pond, and use of local resources can make it more economical [16,17].

Microbial studies in aquaculture are focused on the understanding of symbiotic and antagonist interrelationships of microbes with eukaryotes such as fish, crustacean, and molluscans. Microorganisms of aquatic system were used as biomarkers or sentinel, effluent bioremediators, probiotics and a direct food source for the cultured species [18–20]. Despite such vigorous and constant growth of the use of microorganisms, most of the bacterial species thriving within culture systems and their particular roles in such microsomes are not clear. Determination of metabolic process performed by microbes in aquaculture system is important to point to achieve better understanding. It increases the possibilities of manipulating the microcosms created by aquaculture to understand the biogeochemical cycles of nutrients within and outside of ponds, the modification of bacterial communities and disruption of key processes that lead to disease [21]. Studies on diversity, abundance, symbiotic and antagonist of bacteria in the aquatic system will pave the way to understand and optimize nutrient cycles, disease control, water quality, farming production, the environmental impact of effluents [18–20]. Metagenomics studies allowed researchers to study the diversity and quantity of particular microbes or genes along spatiotemporal patterns to make stronger associations among given microbial communities and host genotype or phenotype [22–24].

It is also well known that, diverse range of microorganisms of biofloc and their cell wall components have been used as probiotics and potent immunostimulants to develop innate immunity, antioxidant status and disease resistance of shrimps against invading pathogens. Microbial components of biofloc also contain potent bioactive compounds including carotenoids, chlorophylls, polysaccharides, phytosterols, taurine and fat-soluble vitamins and shown to be involved in improving the immunity of shrimp species [9,10,16].

There is no information on the role of carbon ratio in improving the microbial diversity in a biofloc based system, growth and immune response of shrimps reared in it. The knowledge of the microbial composition, structure, and stability of a biofloc and its nutritional value results in the improvement of cost-effective shrimp feed preparations. The goal of present study was to evaluate the effects of four different ratios of Carbon and Nitrogen (CN: 5; 10; 15; 20) on growth, and immune status of *L. vannamei* and variation in microbial community in the culture system with zero water exchange.

2. Materials and methods

2.1. Experimental site and tank preparation

The study was carried out for 120 days in the Institute facility. Experimental tank systems of 500 L capacity kept in an open-air structure in a semi-translucent roof. All the tanks were covered with nylon nets to prevent escape of the animals. Tanks were filled with prechlorinated and filtered 32 ppt seawater (sand filtered), 10% of which were regularly exchanged on a fortnight basis. Initially, the tanks were treated with agricultural lime ($CaCO_3$) @ 20 ppm, and inorganic fertilizers like urea (@ 15 ppm) and single superphosphate (@ 15 ppm) to develop the system autotrophically followed by carbohydrate addition for driving the system heterotrophically.

2.2. Experimental design

The experiment was conducted with four different level of C:N ratio (5, 10, 15 and 20) and designated as C:N 5:1, C:N10:1, C:N15:1 and C:N 20:1. All treatments had three replicates and allocation for each treatment was completely randomized to generate the biofloc in all the treatment tanks. Molasses as carbohydrate source (200 ml), probiotics consortium (*Bacillus strains* (5.4×10^9 CFU/ml), were mixed in autoclaved seawater (10 L) and dissolved thoroughly and brewed for 24 h for fermentation. The fermented inoculum was applied in all the treatment tanks @ 50 ml/tank every day for five days to generate the heterotrophic bio-floc. Then C:N ratio was maintained followed by the method of [25] for transition of the heterotrophic system.

2.3. Animal stocking and biofloc management

After the nursery period of 35 days, *L. vannamei* juvenile shrimp with graded weight, 1.0 ± 0.01 g were stocked at 150/m^3 with working volume of 500 L. Formulated pellet feed containing 35% of crude protein (CP) were used as feed in all the treatments. Daily feeding started at 8% of body weight and gradually reduced to 2.5% towards the end of the experiment. The feed was distributed equally to shrimps in all the experimental units, thrice daily at 6:00 a.m., 11:00 a.m. and 6:00 p.m. initially for two months followed by one additional feeding ratio at 10:00 p.m. up to the end of the experiment.

2.3.1. Carbohydrate addition

In this study, the molasses was selected as a carbon source (28% carbon w/w and specific gravity of 1.2). These four different levels of C:N ratios were calculated by C:N contents of the feed and the carbon content of the molasses. The C:N ratio of the applied feed was 7:1 which is adjusted at the base level. A tank without molasses supplementation was referred as a control. To achieve the C:N ratios to C:N 5:1, C:N 10:1, C:N 15:1 and C:N 20:1 above the base level of inherent CN in feed; 0.32 mL, 0.64 mL, 0.96 mL, and 1.28 mL of molasses were added daily for every 1 g of the feed offered, respectively. Control groups was maintained in autotrophic way by developing bloom using above mentioned fertilizers and fed with same feed without addition of any carbon sources. The composition of experimental diet [26] was tabulated in Table 1.

Continuous aeration and agitation were provided by one 5HP blower passing through sand stones aerator, fixed at 10 cm above the ground, with a capacity of injecting 7.5 m^3 air/tank/minute. In the biofloc treatment tanks, minimal water exchange that is up to 10% of water was exchanged in every 15 days interval, and sludge removal was done regularly on a daily basis, whereas for control on a weekly basis 50% of water was exchanged. This was followed throughout the experimental period.

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