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Biofloc-copefloc: A Novel Technology towards Sustained Aquaculture

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Aquaculture being the precious source of fish protein is increasingly practiced worldwide. It offers plenty of opportunities to eradicate poverty, food shortage and malnutrition besides increasing the economic growth and ensures better use of natural resources. It is necessary to increase aquaculture production to meet the increasing demand per capita in parallel to the rise of the global population. Therefore, the aquaculture would provide the most reliable supply of seafood in future. Among the various aquaculture systems, shrimp farming is a rapidly increasing industry that has been supporting the growth and supply of the crustacean-protein to consumers around the world. However, there have been many controversial issues in aquaculture regarding food quality, food nutrition, food safety, and sustainability, that are directly related to the nutrition and feeds for farmed shrimp. There are some issues in the area of shrimp nutrition that require consideration, *viz*., feed and nutrient efficiency, overfeeding and waste, fish meal and fish oil replacement, shrimp and fish health, biotechnology, and human health concerns. The development of aquaculture hatchery and aqua-industry through innovative farming technology is the need of the hour. The new concept via novel technology has been introduced in the shrimp farming sector too where the farmers are building copefloc instead of biofloc to stimulate the production of crustacean copepods in the system. The copefloc would not only act as excellent natural food but also act as an immunostimulant to keep the diseases at bay. The perspective, manner of copefloc technique (CFT) is considered as a promising novel technology for the aquaculture system. Copefloc is used as new live-food in larviculture of shrimp culture industry in Thailand for the first time in the world. This new Biofloc technology-based floc particles are being used as main live food for culturing shrimps, and not for use the food industries. BFT and CFT of recirculation aquaculture system (RAS) is the most advanced technology to the shrimp farming industry that provides natural live feed "Copepod" for post larvae (PL), prior to stocking in ponds. That would enhance the water stability, maintain good survival rate (SR), promote fastest growth rate (GR) and high profitability while making it totally sustainable without any negative impact to our environment. This paper reviews the advantages of using a combined BFT and CFT, a novel technology system in sustainable shrimp farming.

(***Key words:*** *Amphipod, Biofloc,Copefloc, Copepod, Microalgae, Probiotics)*

Since the past decade, production of finfish and shellfish via, aquaculture practices have exposed incredible growth potential and its promise being the world's leading food-producing capacity. During the past, the shrimp farming industry in some asian countries have suffered from environmental issues and virtually collapsed almost all and solution has been found so far. Since the 1990s, aquaculture has become the fastest-growing animal food-producing industry in the world (FAO, 2014). Globally farmed shrimp production reached almost 4 million tonnes in 2018, increasing by 3 to 5% over 2017. China's negative production trend for marine shrimp got reversed and production increased by 10% in 2018 (FAO, 2019). Import prices fell to a record low level but with minor improvement in imports to the conventional developed markets. Strong buying by Asian markets, particularly China, saved the shrimp aquaculture industry worldwide from a major and far-reaching financial crisis in 2018 (FAO, 2019).

Aquaculture has been one of the most promising animal food-producing sectors, with the lowest feed conversion ratio (Smil, 2011), and which provides sufficient and highly nutritional food for a growing world population. Particularly, the industry would play a crucial role in meeting increasing demands for healthier animal proteins and lipids. Thus, the contribution of aquaculture in alleviating obesity and its resultant health and social benefits is clear and should not be underestimated (Santhanam *et al*., 2019). However, despite these positive attributes and success stories, the

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aquaculture industry has also faced many challenges, including environmental issues (Asche *et al*., 1999), fish meal (feed) problems (Naylor *et al*., 2000), disease outbreaks (Asche *et al*., 2009) and price volatility (Oglend, 2013). If aquaculture is to live up to its promises, it must follow a sustainable production line. Among other things, sustainability should be achieved in an economically efficient and cost-effective way. To this end, potential barriers should be identified throughout the entire production cycle, and better practices and innovations have to be implemented accordingly.

The aquaculture industry is one of the sub-sectors that requires substantial improvement in nutrition and feeds for farmed fish and shrimp. These nutrition-related issues that must be considered in order to achieve balanced and safe nutritious food production and sustainability in aquaculture. The better practices and innovations in larval rearing are particularly necessary for major upscaling in the production of existing species and efficacious farming of "new species" (Dhert *et al*., 2001; Mahjoub *et al*., 2013; Santhanam *et al*., 2019). The early stage of larval rearing is the most important as a backbone of the aquaculture hatchery and aqua-industry. The live feeds are crucial given the fact that the lion's share of commercially farmed species (such as turbot, shrimp and grouper) require live feeds for the first feeding (Mahjoub *et al*., 2013; Santhanam *et al*., 2019).

Feeding the first/early larval stages of aquatic organisms appears to be the major constraint in aquaculture (Agh and Sorgeloos, 2005). Live food organisms contain all the nutrients such as proteins, lipids, carbohydrates, vitamins, minerals, amino acids and fatty acids and hence live feeds are popularly known as "living capsules of nutrition" (Tiwari, 1986; Manickam *et al*., 2017). Suitable live food plays a most important role in achieving maximum growth and survival of the juvenile finfishes and shellfishes. The size of cultivated live food (for their larvae) plays a key role. Also, live foods organisms slowly swim in the water column and are constantly available to finfish and shellfish larvae (David, 2003). Therefore, to sustain productivity we have to introduce "new" farmed species in the aquaculture industry, aqua-hatchery and aqua-farms for the larval rearing stage. To this end, the focus ought to be on live feeds, as the success of larval feeding relies directly on improvements in live feeds.

Copefloc general information

Copefloc is the most advanced technology to the shrimp farming industry that provide natural live diets that is "Copepod". Biofloc-copefloc technology is well accepted and highly essential in the current scenario for various reasons, such as to produce more aquaculture products without significantly increasing the usage of the basic natural resources of water and land. To develop sustainable aquaculture systems that will not damage the environment and providing an equitable cost/benefit ratio to support economic and social sustainability. The copefloc technology, similar to biofloc, has many more advantages, such as 1. Free ammonia (NH_3) in water (H_2O) is converted into protein in heterotrophic microbial biomass, collected into suspended biofloc particles in water; 2. Improve the level of biosecurity, reduce the risk of infection by not having to change water; 3. Does not pollute the environment; reduce the cost of food, medicine, chemicals. Biofloc is the use of beneficial microorganisms (probiotic bacteria) as a floc in the aquaculture systems to maintain water quality and to achieve production goals. In any of aquaculture practices, the operating bio-filter and mechanical filtration systems require complex equipment systems, high operating costs, and high technical capabilities. However, fortunately the copefloc system, is capable of improving the environment as they are natural organisms and creating natural food biomass, contributing the nutrients through their feces which is serving as medium microalgae to grow. This operation is also quite simple. As the copefloc technology requires absolutely no use of chemicals or antibiotics, there will be an improvement in the quality of products and goods.

Biofloc to copefloc: technological system

The biofloc technology (BFT) farming uses microorganisms in production systems to incorporate waste nitrogen to feeding. It is an innovative and cost-effective technology in which toxic materials to the finfish and shellfish such as nitrate, nitrite and ammonia can be converted to a useful product, *i.e*., proteinaceous feed. It is the technology used in the aquaculture systems with limited or zero water exchange under high stocking density, strong aeration and the biota formed by biofloc. The culture of biofloc will be productive in the case of culture tanks exposed to the sun. A novel biofloc system was developed to improve the environment control over

the aquatic animal production. The aquaculture, strong influential factors are the feed cost (accounting to 60% of the total production cost) and the most limiting factor is the water/land availability. High stocking density and rearing of aquatic animals require wastewater treatment. Biofloc system involves wastewater treatment that has gained vital importance as an approach in aquaculture. Microorganisms will need nitrogen and carbon as nutrition to grow and reproduce, and their biomass encapsulates the nitrogen dissolved in the water. The biofloc system lacks sufficient carbon and needs to be added the carbohydrates such as tapioca, sugar or molasses for beneficial bacteria to grow and reproduce. When the bacteria and other microorganisms grow to a certain density, flocs appear in the water that may be called as "biofloc".

The core principle of the biofloc technique is the generation of nitrogen cycle by maintaining a higher C: N (carbon and nitrogen) ratio by stimulating heterotrophic microbial growth, which assimilates the nitrogenous waste that can be exploited by the live-feed species while in culture. The biofloc technology is not only effective in treating the waste but also forms nutrition to the aquatic animal. During the process of addition of carbohydrate source (molasses), higher level of C:N is maintained and the water quality is improved through the production of high-quality single-cell microbial protein. In such a provision, dense microorganisms develop and function both as bioreactor controlling water quality and protein food source. The immobilization of toxic nitrogen species occurs more rapidly in bioflocs because the growth rate and microbial production per unit substrate of heterotrophs are ten-times greater than that of the autotrophic nitrifying bacteria. The technology is based on the principle of flocculation within the system which has been implemented in shrimp farming due to its bottom-dwelling habit and resistance to environmental changes.

Biofloc has the advantage of recreating leftovers as feed for shrimp, less changing of water and limiting the spread of pathogens into the farming system. However, biofloc technology also has some limitations such as continuous aeration requirement to keep the waste floating. In intensive systems, improper placement of aerators also produces strong water currents, which reduces the structural integrity of the floc particles (Crab *et al*., 2012). Further, the decrease in pH and alkalinity due to nitrification with the addition of sufficient carbon are factors that need to be closely monitored and compared to the other shrimp farming methods (Thong, 2014). Other drawback of biofloc system is that it requires constant aeration to keep the wastes in suspension for proper degradation which adds up to the production cost. By considering these loopholes, a novel technology called the copefloc technology has been initiated in the shrimp farming industry which negates the used of feed or any oxygenation in the ponds (Romano and Kumar, 2017).

Food spring for shrimp and shrimp farming

The feeds used in this system mainly are grain and natural food items like microalgae, crustacean copepods and amphipods. These live feeds are nutritious and very good for the growth of shrimp. The copepods are very important food for the larviculture and pond culture of fin and shell fishes and biofloc associated microbes could transform organic wastes into higher protein content in the food protein industry.

Mapping process of food for shrimp in copefloc system

The natural live copepods are inoculated into the culture-ponds with a depth range of 1.2 to 1.5 m. where the oxygen (O_2) enrichment is done by strong aeration for 24 to 48 h. The rice bran at 300 kg ha^{-1} is fermented with probiotics addition in a tubular cheesecloth bag at the bottom of the pond. The continuous aeration is given for 7 to 10 days to develop the favourable environment to form a mass of live copepods in the pond. This copepod-biofloc primer solution is prepared by fermenting the mixture at a ratio of 1 L of water, 10 g cereal (fishmeal, soybean meal, groundnut oil cake, wheat bran, tapioca flour, etc.) and 10 mL of the cultured pure strain of probiotic species *Bacillus subtilis* and *Bacillus sphaericus* with bacterial density of 10^6 mL^{-1}. This fermentation is performed under the fine conditions of strong aeration for 48 h and by maintaining the pH at 6.0 to 7.2 and by the addition of a buffer pH solution, at the range of temperatures 25 to 28°C. Thereafter microorganisms increase, large bubbles floating on the surface is formed and biofloc bait is added to the pond.

Culture of probiotic bacteria

The probiotic bacteria *B. subtilis* and *B. sphaericus* are cultured according to the protocols of Zokaeifar *et al*. (2012) and Santhanam *et al.* (2019). Beneficial bacteria

of *B. subtilis* and *B. sphaericus* are grown in lysogeny broth using an orbital shaking incubator at 150 rpm at the temperature of 30°C for 48 h. These cultures are centrifuged at 3000 rpm × g for 10 min at 4°C and, after discarding the upper part of supernatant, the pelleted bacteria is re-suspended and washed thrice in sterile normal saline solution (NSS of 0.9% NaCl). Thereafter, the cell densities of the suspensions are calculated using a spectrophotometer through absorbance measurement at 600 nm and also correlated to the colony-forming units (CFUs) using the spread-plate technique. The obtained suspension is used as the inoculum for the mass culture in the shrimp pond.

Culture of microalgae

The culture of microalgae is done as per the method of Perumal *et al*. (2015). The stock cultures of microalgae are to be kept in 1 and 2 L culture flasks, and 5 and 15 L plastic containers in a special air-conditioned room. The water is filtered by using a filter bag (1 micron), sterilized through an autoclave and, after cooling, transferred to the culture flasks. The culture flasks are plugged with cotton or covered by aluminum foil. All the vessels used for algal culture are sterilized using an autoclave and dried in an oven before use.

The culture medium used for indoor stock culture of microalgae is selected depending on the species. About 10 mL of the inoculum (in the growing phase of algae) is transferred to the culture flasks and the culture is provided with 1000 Lux light and 12:12 h light and dark cycle. After 8 to 10 days, the maximum exponential phase is obtained. Temperature and salinity are maintained between 23 and 25 °C and 28 and 30 ppt, respectively, for the entire mass culture period. The continuous aeration is provided for the culture. After 8 to 10 days, while the maximum exponential phase is reached, light is reduced to 500 Lux for further growth. The time required for the maximum cell densities varies depending on the species. Under controlled conditions of light and temperature (with or without aeration), the algae is grown. At the time of the maximum exponential phase of growth, the colour of the culture turns dark green.

The mass culture of microalgae is done in fiberglass tanks. For the efficient growth of algae commercial fertilizers namely ammonium sulphate ($(NH_4)_2SO_4$), super-phosphate ($Ca(H_2PO_4)_2$) and urea (CH_4N_2O) at a ratio of 10:1:1 are added to the fiberglass tank. The 100 L of water, monoculture of microalgae (*Chlorella marina, Dunaliella salina, Isochrysis galbana and Nannochloropsis sp*.) at 2 L of inoculum are added to the culture tank. A continuous and vigorous aeration is provided to the culture to keep the culture always in suspension. The stock cultures of microalgae are maintained separately in air-conditioned room fertilized by using Conway's medium.

Laboratory culture of copepods

The culture of copepods is made as described by Santhanam *et al*. (2015 and 2019). The copepods samples are to be collected from brackish water and other marine environments using plankton net with mesh size 158 µm. The collected samples are taken to the laboratory and zooplankton samples are screened by using a set of superimposed sieves of varying mesh sizes, with decreasing mesh size from upstream to downstream. Zooplankton samples are screened coarsely through a 500-µm mesh to remove finfish and shellfish larvae. After that, the samples are screened through a 190-µm mesh to remove the rotifers and copepods nauplii. Finally, the samples containing predominantly adult, late-stage copepodids and egg-bearing female copepods are isolated by using a fine brush, needles and a Stempel pipette under a stereoscopic microscope.

The adult-female copepods are selected for the purpose of making the culture monotonous and free of contamination from other copepods. The known number of copepods, including males and females or gravid females, are stocked initially in 250-mL glass beakers and conical flasks provided with cultured microalgae without aeration. Later, copepods are sub-cultured into 7 L plastic containers filled with filtered water and vigorous aeration is provided. After that, the copepods are transferred to an oval-shaped, flat-bottomed fiberglass tank filled with 100 L of filtered water and vigorous aeration is invoked for mass scale culture. The water quality parameters, such as temperature, salinity, pH and dissolved oxygen, are maintained in the ranges; 26-30°C, 28-32‰, 7.5-8.5 and 5.0-7.5 mL L^{-1}, respectively. Marine copepods are fed with a daily ration of mixed microalgae, *viz*. *C. marina, D. salina, I. galbana and Nannochloropsis sp*., at a concentration of 25,000 cells mL^{-1}.

Large-scale culture of copepods

The large scale (pilot-scale) culture of copepod is done by using different capacity Fibre-reinforced plastic (FRP) tanks. The FRP tanks are washed with a low-residue laboratory detergent (e.g. Alconox or Sparkleen) and water, followed by thorough rinsing. Then the tanks are treated with 100% muriatic acid (HCl) solution, followed by thorough rinsing with filtered water. The FRP tanks are leached three times (24 h each time) to remove all water-soluble remnants of the manufacturing process. The FRP tanks are filled to the rim with filtered, UV-treated water and the salinity is adjusted as needed, and water is chlorinated with 60 mL (0.2 mL L^{-1}) commercial 10% hypochlorite solution per liter. The treated system is allowed to stand for 24 h. After that, the system is de-chlorinated with 60 mL of stock thiosulfate solution. The vigorous aeration is then started. After an hour, a "free chlorine" test strip is dipped and zero "free chlorine" remaining is ascertained. The treated water is aerated to at least 6 mg L^{-1} dissolved oxygen (DO). The water conditions, *viz*; salinity, pH, DO, colour and scent (particularly the "rotten egg" smell of H_2S), are checked prior to collection and treatment. The water is serially filtered through 50, 10 and 1-µ m mesh bags followed by sand filter, carbon filter, biological filter and then passed through the ultraviolet sterilizer. The filter bags are cleaned and then sanitized overnight in hypochlorite solution once a week under normal usage. The filtered; UV-treated water is used for the culture of copepods. Filtered water is treated with 10% commercial hypochlorite solution at 0.2 mL L^{-1} and left to stand overnight without aeration. Then, the water is dechlorinated with thiosulfate solution volume for volume (v/v) at 0.2 mL L^{-1}. The dechlorinated water is used for filling all wash bottles, stacked-sieve holders. The cultured copepods are to be harvested and population counts are made using the counting chamber.

Large-scale culture of copepods has to be started with a clean FRP tank, microalgae and filtered, UV-treated water. The FRP tanks are stocked with a known number of gravid females. Gravid females would release the nauplii within 36 to 42 h. The debris is removed daily by using graded sieves connected with a siphon hose, and then adults and nauplii are returned to the tank. The sequential batch cultures are to be started at 5 to 7-day intervals for the continuous copepods production. The cool; filtered, interior air is used for aeration. The disposable inline 0.2 µm-pore antibacterial air filters are used for all aeration. The aeration is used to maintain algae culture, CO_2 saturation, pH stability and for uniform mixing. A stacked-sieve holder and wash bottles are washed with treated seawater at the culture tank temperature. The siphon hose is connected to the siphon head and the stacked sieves. The copepods are harvested and filtered onto a wet free-standing sieve. As the tank's water level drops, frequent rinsing is given to remove the copepods stuck to the sidewalls. Thirty per cent of the tank volume is exchanged weekly with new treated water. From these stock cultures of copepods in large FRP tanks, the copepods are harvested and then introduced to the shrimp culture tanks and ponds for the move towards biofloc-copefloc technology approach.

Culture of amphipods

The amphipods culture is performed according to the protocol of Baeza-Rojano *et al*. (2013). Amphipods are collected from the estuarine, coastal intertidal, mangrove and backwater areas. The individuals are isolated from the sediment samples and are transported with aeration, together with some sediment or oyster shells, to the laboratory. The cylindrical FRP tanks of 100 L capacity are used for amphipod culture. FRP tanks are supplied with running seawater, with a complete water replacement of the tank every hour. The salinity and dissolved oxygen are maintained in the range of 37 to 39 g L^{-1} and 5 to 9 ppm respectively. The natural water temperature fluctuation (25 ± 3 °C) and a natural photoperiod, 14:10 h for light/dark are maintained. During the experimental period, the amphipods are daily fed with *Artemia nauplii* (500 mL of seawater with a density of 1400 nauplii mL^{-1}) and a mixture of microalgae *viz*; *I. galbana and Tetraselmis suecica* (2 L of seawater with mean densities of 11.9×10^6 cells L^{-1} and 2.9×10^6 cells L^{-1}, respectively). *Artemia nauplii* is previously enriched with a synthetic enrichment market product and, when the *Artemia* and microalgae are added to the tanks, the water inflow is reduced so as to increase their residence time (Nakajima and Takeuchi, 2008). Inside the culture tank, three different plastic meshes of dimensions 30 × 50 cm are used as an artificial substratum for the amphipods. The culture is started with a known number of brooders added to the tank (e.g. 125 females and 125 males). Three months after the introduction of the brooders to the tank, the plastic meshes containing amphipods are recovered

individually. Amphipods are mass cultured in larger FRP tanks and the cultured ones are introduced to the shrimp culture tanks and ponds towards the biofloc-copefloc technology process

Biofloc-copefloc system operations

The biofloc-copefloc technology is performed according to the protocols of Avnimelech (2009), Crab *et al*. (2012), Romano and Kumar (2017) and Santhanam *et al*. (2019). In aquaculture industry, the development of new innovative and novel system of biofloc-copefloc technology does not create any waste, so there is no need to pump the system for waste material to collection convergence. As an alternative, the aeration system layout needs to supply more oxygen to the shrimp, microorganisms and natural food in the pond. Copefloc farming technology uses a bottom aeration system which is rash with small holes drilled on the flank of the PVC pipe, with space between the holes at the range of 25 to 30 cm, thus forming a network with an area covering about 40% of the total area of ponds/tanks (instead of using very little aeration/water fan system). The stocking density is less than 50 nos. m^{-2}. The shrimp grows well and it completely uses the natural food. As an alternative, farmers have to manage and maintain the population of natural food, the biofloc volume in the pond/tanks. The copepods are collected and calculated for their density by using buckets, 50 to 100 L of water at different positions in the pond/tanks are collected and then filtered through plankton nets with mesh size in the range 50 to 70 µm, added into 600 mL vials and formalin fixed at 2 to 4%. l ml of copepod sample is pipetted and counted under a microscope at magnifications of 10X and 40X (by moving the chamber counting method by coordinates). After that, the density of copepods are computed and the required amount of probiotics bacteria applied to the pond for feeding copepods. For the formation and maintenance of biofloc stability, a biofloc carbon source primer is to be supplemented and complement the culture system. There are many sources of carbon like cereals, molasses, bagasse pulp and straw, grass that can be used. The concentration of biofloc should be maintained at <1 mL L^{-1} during the production cycle (Fig. 1).

The copefloc technology as well as biofloc, has many added advantages in the aquaculture shrimp farming. Copefloc technology is considered to be an efficient alternative system since nutrients could be continuously recycled and reused. The step 1, ammonia (NH_3) in water is converted into biomass protein by heterotopic microorganisms, gathered into biofloc

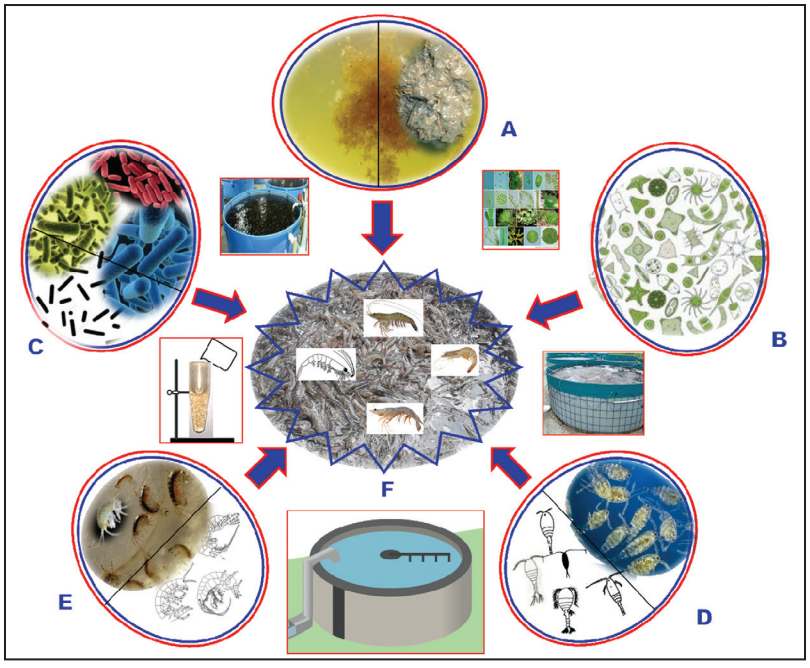

Fig. 1. *Principle of biofloc-copefloc shrimp farming*

A. Grains; B. Microalgae; C. Probiotic Bacteria; D. Copepods; E. Amphipods; F. Copefloc cultivable Shrimp

particles suspended in water; step 2, an improvement in the level of bio-security, reduction of the risk of infection due to water changes; step 3, they do not pollute the environment and reduce the cost of food, drugs and chemicals and also shrimp grow faster in farming. The technology of the design and operation of copefloc is very simple and absolutely no more food industrial use, can put the shrimp PL10-12 directly into the pond (without acclimatization). As this technology involves biofloc as water quality manager and copefloc as natural feed, there is no need for the periodic water exchange (as it would increase the energy costs and also increases the risk of infectious pathogens from water supplies of pond). Also, there is no need for any kind of filter system, which requires complex equipment, high operating costs and technical capabilities. The new copefloc system is able to improve the environment as it creates natural food biomass (live feed) and contributes reusable waste nutrients from aquatic animals; it is a practically simple operation. The novel copefloc technology has completely obviated the use of chemicals of antibiotics, thus improving the quality of products and goods.

A novel biofloc-copefloc technology has increased the attention of aqua farmers nowadays as a sustainable intensive shrimp farming technology. The high stocking densities are now possible with this technique, which combines biofloc-copefloc technology with natural live feed production in ponds or tanks. Thus, the biofloc and copefloc technology is considered to be an environmentally friendly way of farming as it involves natural feeding system and not industrial food used. Therefore, the extensive aquaculture practices would be followed by adopting this BFT-CFT that would pave a way towards sustainable aquaculture production.

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