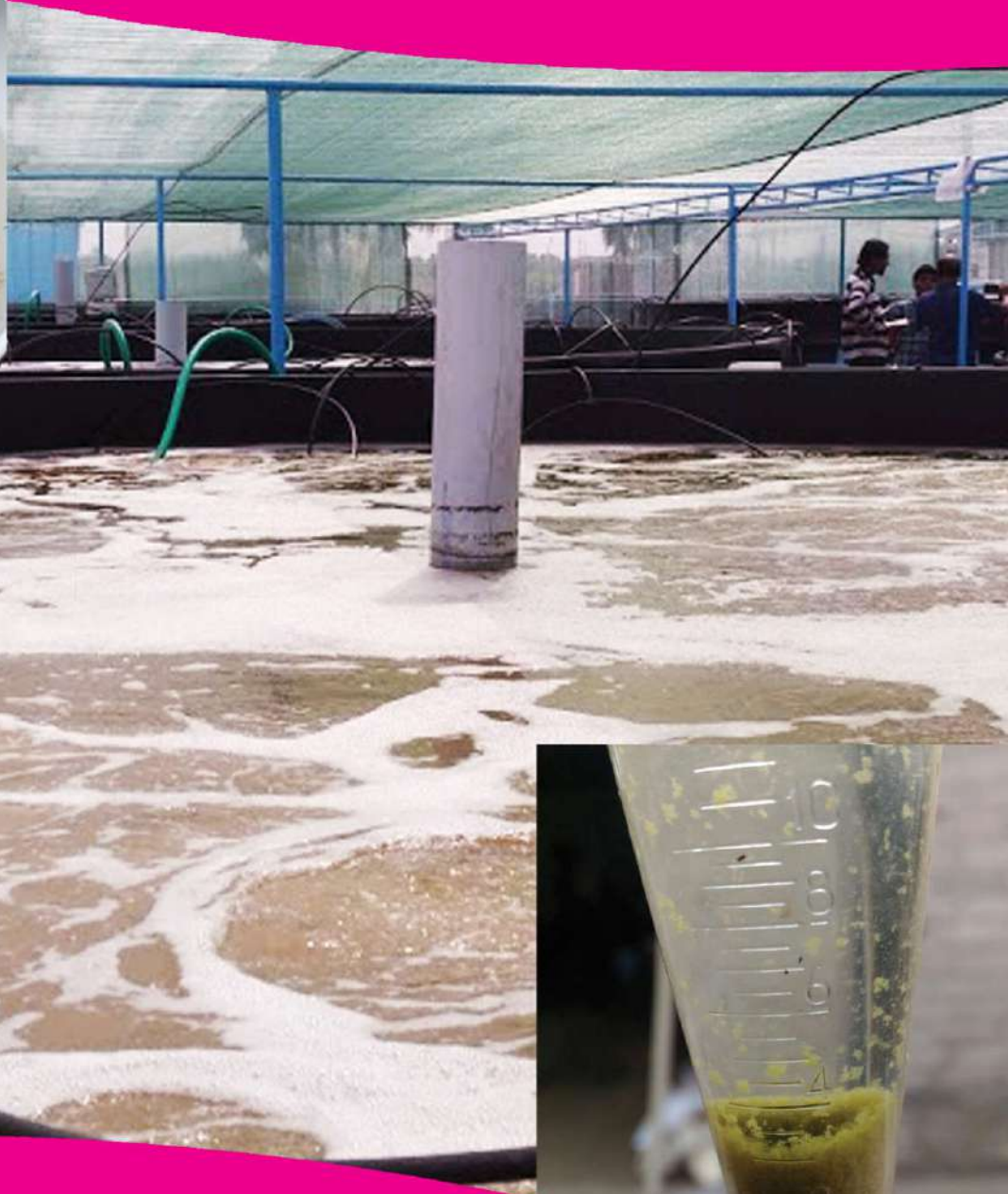




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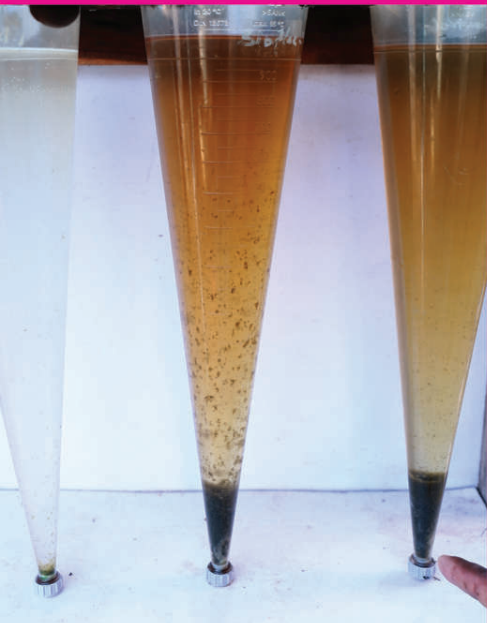
BIOFLOC TECHNOLOGY FOR NURSERY AND GROWOUT AQUACULTURE



09-12 Nov 2021

Organized by

ICAR - Central Institute of Brackishwater Aquaculture (CIBA)
Chennai 600028, Tamil Nadu







BRACKISHWATER AQUACULTURE FOR FOOD, EMPLOYMENT AND PROSPERITY

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Training manual on
**Biofloc Technology for Nursery and
Growout Aquaculture**

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Published by

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Training manual: Biofloc Technology for Nursery and Growout Aquaculture

Brackishwater aquaculture for food, employment and prosperity

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INNOVATIVE CULTURE PRACTICES IN BRACKISHWATER AQUACULTURE WITH ECOSYSTEM APPROACH

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Brackishwater ecosystem has wide range of seafood species such as fish, shrimp, crab, seaweed, micro algae etc. with tremendous nutritional value as well as nutraceutical properties and in India, it has evolved as a commercial enterprise with an impressive annual growth rate of 6-7 percent. Out of total 3.9 million ha of the estuarine area estimated in India, 1.2 million hectares of coastal saline lands have been identified to be potentially suitable for brackish water farming. Only 15% of the potential brackishwater area is utilized for farming which shows there is a tremendous scope for further expansion. Also, about 9 million hectares of salt-affected lands were assessed in the hot semi-arid and arid ecoregion of northern plains and central highlands. Brackishwater aquaculture is a “sun rise sector” in India, which plays a crucial role in socio-economic expansion and is considered as influential income and employment generator. In this journey of increasing overall production in the sector, more than twenty five fold from 28000 tons in 1988-89 to 7,47,694 tons in 2019-20, the most important turning point was the introduction and successful establishment of SPF (Specific Pathogen Free) *P. vannamei* in 2009. Also recently revival of monodon through SPF *P. monodon* introduction in 2020-21 is getting attention. Along with intensification, disease outbreaks, environmental degradation, eutrophication, traceability, antibiotic issue, biodiversity threat, the escalating price of feed ingredients, other accessory inputs cost, etc., became daunting challenges in BW aquaculture. However, along with intensification of commercial shrimp culture, industry started to face issues like spiralling price of commercial feed, diseases outbreaks, sustainability concern etc. Hence, the concepts of delivering high production with sustainable approach through evolving eco-friendly technologies started getting momentum worldwide. Modern, industrial aquaculture could strengthen its social and ecological roots by articulating its evolution along a sustainability trajectory and by adopting fully the Food and Agriculture Organization (FAO) ecosystems approach to aquaculture (EAA; Soto *et al.*, 2007).

Ecosystem approach to aquaculture (EAA) *is a strategy for the integration of the activity within the wider ecosystem in such a way that it promotes sustainable development, equity, and resilience of interlinked social and ecological systems*” (Soto *et al.*, 2007). The present article provides innovative aquaculture practices for Indian brackishwater aquaculture keeping in mind the ecosystem approach, its principle, relevance and conceptual framework.

Ecosystem approach to aquaculture (EAA)

The Fisheries and aquaculture department of FAO recognized the need of development of ecosystem based management for aquaculture in the line of the code of conduct for responsible fisheries in the year 2006. EAA is defined as “a strategy for the integration of aquaculture with the wider ecosystem such that it promotes sustainable development, equity

and resilience of interlinked social-ecological systems”. FAO suggested three objectives for the ecosystem approach of aquaculture: human wellbeing, ecological wellbeing and ability to achieve these by effective governance, and these can be measured at farm, region and global level. EAA works on three interlinked principles. These three principles are operated at three levels, farm level, watershed or region level and globe level (Figure 1 a & b). These three principles are:

Principle 1 “*Aquaculture development and management should take account of the full range of ecosystem functions and services, and should not threaten the sustained delivery of these to society*”

Development of aquaculture within the acceptable limits of environmental variable requires an understanding about the carrying capacity of the ecosystem and ecosystem functioning. Any aquaculture pond or cage is the ‘aquaculture ecosystem’ and the ecosystem where this production system is embedded is the wider ecosystem. The resilient or carrying capacity of this ecosystem should be defined.

Principle 2 “*Aquaculture should improve human well-being and equity for all relevant Stakeholders*”

Aquaculture should promote food security and environmental safety. Here food security does not suggest that it should solve the problems of hunger, particularly area where aquaculture is a new activity. However, it should promote livelihood and generate employment opportunity. Aquaculture development should ensure that its benefits are properly shared among all the stakeholders.

Principle 3 “*Aquaculture should be developed in the context of other sectors, policies and goals*”

This principle acknowledges the opportunity of integrating or linking aquaculture with other producing sector to promote material and energy recycling and optimal use of resources. Aquaculture does not take place in isolation, although its impact to other human activities is rather lesser than agriculture and industry.

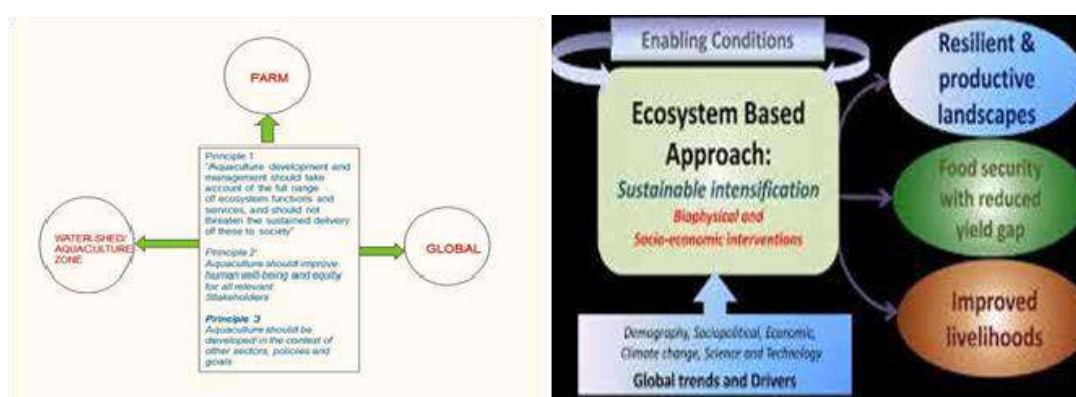


Figure: a) Ecosystem based approach to aquaculture-guiding principles and scales.
b) Sustainable intensification.

Ecosystem approach to brackishwater aquaculture (EABA) in India

Considering the full range of ecosystem functions and management has traditionally been practiced in brackishwater aquaculture in India, which is closely associated with the principle one. Further, there has been research initiation to refine the technique and document the current practices. In this system effluents and residues from the farming system has been recycled and used as resource.

Traditional brackishwater aquaculture system in India

In the coastal states like Kerala, West Bengal, Karnataka and Goa, traditional brackishwater aquaculture prevails which are classical examples of integrated aquaculture, essentially falls under the framework of EABA. It is practiced in two forms 1) Temporal integration of rice with shrimp 2) Simultaneous integration of rice and fish culture. In this type of system, tall, salt tolerant rice varieties are cultured during the monsoon season (summer monsoon: June to Nov) in the fields bordering the backwaters of Kerala, and during the post monsoon and summer season shrimps are cultivated. In the later during the rainy season when salinity is negligible, rice and brackishwater fishes are cultivated simultaneously. Chemical fertilizers or pesticides are not used.

The economic return of rice-fish/shrimp integrated system indicates that rice and fish followed by shrimp provides significantly high economic returns. Presently, the traditional system is modified by stocking with hatchery reared seed and supplementary feeding. The recent research also attempts to use improved saline tolerant rice varieties to circumvent the low productivity of traditional rice varieties, to enable increased economic returns to the farmer. The availability of hatchery produced seeds of penaeid shrimps and fin fishes such as sea bass, pearl spot and increasing knowledge about this ecosystem provides an opportunity to optimize the sustainability and economic viability of this type of farming practices.

Research efforts at CIBA

Over the years, ICAR-CIBA has generated significant information on shrimp, fish, crab hatchery and grow-out production, nutrition and feed-technology, disease diagnosis and management to address the growing needs of brackishwater aquaculture sector and provided a platform for interaction with stakeholders. These technologies have the ecosystem approach based footprints and are discussed here.

Polyculture based production system

ICAR-CIBA carried out several experiments to evaluate the production potential of polyculture of brackishwater fin fishes and shell fishes. In an experiment to evaluate the polyculture in an extensive system, farm level performance of two systems were evaluated: shrimp with mullets (*Mugil cephalus*, *Liza parsia* and *L. tade*), and shrimp milk fish (*Chanos chanos*). In the 180 day culture experiments, it was found that the production is similar in both systems. However, tiger shrimp out performed in mullet-shrimp system than the milk fish shrimp system. It indicates that the mullet is more compatible with shrimp than milk fish. Further, this study also concludes that resource poor farmers can adopt this system as the input cost and expenditure is low.

Organic production system for brackishwater species

Organic aquaculture is a process of production of aquatic plants and animals with the use of only organic inputs in terms of seeds, supply of nutrients and management of diseases. Organic production system is an ecosystem based approach to aquaculture. Organic foods have a separate niche market and many farmers are attracted to these farming practices due to lower cost of production and better economic returns. In India, INDOCERT provides certification for organic production systems. Although organic aquaculture is in a very nascent stage in India, its traditional system is close to the organic way of farming.

Organic Aquaculture: periphyton based farming

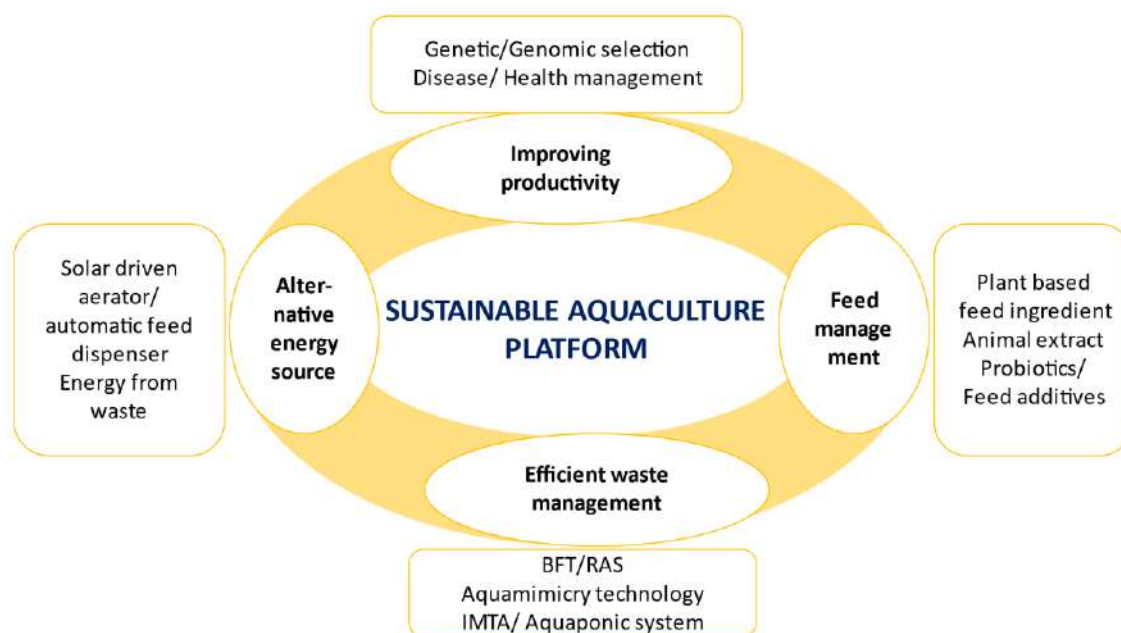
CIBA has attempted research effort to enhance the production and sustainability of shrimp farming within the frame work of EABA. Periphyton based farming is an attempt in this direction. Periphyton refers to the entire complex of attached aquatic biota on submerged substrates comprising phytoplankton, zooplankton, benthic organisms and detritus. The study conducted by CIBA clearly indicates that periphyton has a beneficial effect on growth and production of shrimp. Better growth rate with a productivity of 1640 to 2796 kg/ha/crop at a stocking density 8-12 individuals/m² was observed. Further, the rate of return over operational cost was higher in periphyton-based system (92%) compared to the conventional farming (54%). This level of improvement of pond production with cheap on farm resources enhance the productivity of shrimp ponds without deteriorating ecosystem.

Integrated multi-tropic Aquaculture (IMTA)

Integrated Multi-Tropic Aquaculture is the farming of different aquaculture species together in a way that allows one species' wastes to be utilized as feed for another. Farmers can combine fed aquaculture (e.g., fish, shrimp) with inorganic extractive (e.g., seaweed) and organic extractive (e.g., shellfish) aquaculture to create balanced systems for environment remediation (biomitigation), economic stability (improved output, lower cost, product diversification and risk reduction) and social acceptability (better management practices) (Barrington *et al.*, 2009). This forming model can be developed for augmenting the average productivity of open waterbodies.

Bio secured zero water exchange shrimp farming technology (BZEST)

Bio secure zero-exchange system for shrimp represents an emerging technology that provides a high degree of pathogen exclusion with minimal or zero water exchange. This zero water exchange shrimp farming system is an evolving culture practice with use of probiotics (Panigrahi, *et al.* 2007) and zero tolerance to banned chemicals and antibiotics. CIBA has developed a BZEST for application in the shrimp farming sector, which is characterized by the improved productivity and better FCR. This BZEST system is amenable for control of disease through Best Management Practices and preservation of water resources.



Bio-floc based technology for brackishwater species

This is a relatively new technology to support high density, better water quality, water conservation, bio security, lower feed requirement and reduce the production cost. The concept of biofloc technology work around the formation of dense heterotrophic bacterial community. Eventually the system becomes bacterial dominated rather than algae dominated and forms microbial flocs by utilizing the waste materials in the pond. Biofloc is the conglomeration of micro organisms (such as heterotrophic bacteria, algae (dinoflagellates & diatoms), fungi, ciliates, flagellates, rotifers, nematodes, metazoans & detritus). Constant aeration and intermittent addition of carbon source as organic matter for the bacteria is needed to prevent the collapse of the system.

In a typical brackishwater pond, 20–25% of fed protein is retained in the fish/shrimp, rest is wasted as ammonia and other metabolites. Manipulating the C:N ratio in the pond enhances conversion of toxic nitrogenous wastes into microbial biomass available as food for culture animals. CIBA has initiated efforts to develop a biofloc model suitable for Indian brackishwater farming systems. A series of experiments in pilot scale was conducted at CIBA showed measurable gain in the production as well as FCR in tiger shrimp *P. monodon* farming by following these eco based techniques (Shyne *et al.* 2012). Several studies (Panigrahi *etal.*, 2014; Sujeet *etal.*, 2015) indicates that bio-floc with periphyton systems (BPT) increased growth, survival and protective response.

Seaweed integration with brackishwater aquaculture species

There are attempts from research organization as well as private sectors to integrate shrimp aquaculture with seaweeds to make the intensive aquaculture more environmentally non degradable. Pacific Reef Fisheries, Pvt Ltd. started growing sea weed, *Ulva* spp in the 5 ha race way of their 98 ha *P. monodon* farm, and reported that this would be sufficient to remove the Nitrogen and Phosphorous from the effluent water from the shrimp farm. Further, the

secondary crop provides additional income. CIBA have initiated research in this direction developing model farming with seaweed integration.

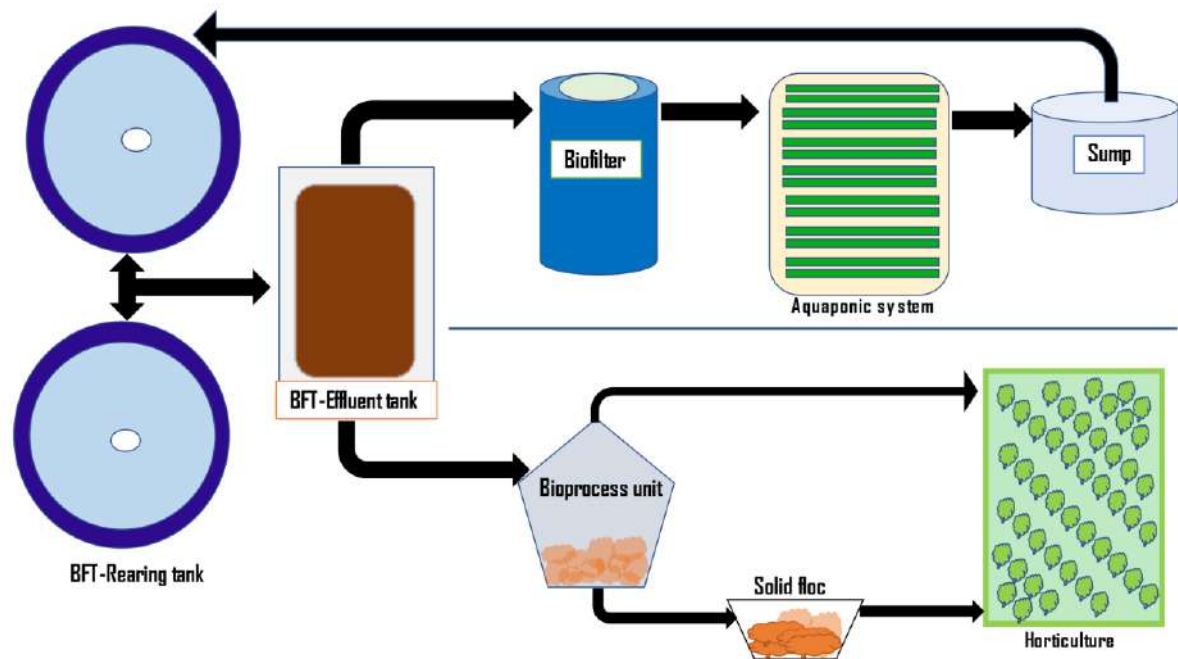


Fig. illustrating Aquaponics integrated with BFT system

Carrying capacity

Carrying capacity is the major component of the EABA that helps to set upper limits of aquaculture production within the limits of environment or ecosystem and social acceptability (Ross *et al.*, 2013). Carrying capacity is defined as “*In general terms, carrying capacity for any sector can be defined as the level of resource use both by humans or animals that can be sustained over the long term by the natural regenerative power of the environment*” (FAO, 2010). Aquaculture is a resource based industry, and therefore, it will compete with other allied industries, for example, fisheries, agriculture and tourism. It is therefore, essential to determine the carrying capacity for the sustainable development of aquaculture. Carrying capacity has been categorized into four: physical, production, ecological and social (McKindsey *et al.*, 2006). 1) Physical carrying capacity quantifies the potential area available for aquaculture in the ecosystem. 2) Production carrying capacity estimates the maximum aquaculture production.

3) Ecological carrying capacity determines the magnitude of aquaculture production without leading to the detrimental changes to the ecosystem. 4) Social carrying capacity is the amount of aquaculture that can be developed without major environmental and social impacts. CIBA has developed decision support software in visual basic to estimate the maximum allowable farming area for a particular creek or drainage canal (Muralidhar, 2009). This software helps to determine are liable estimation of impact of shrimp farming and other land use impact in a region under various scenarios of increased development.

Conclusion

CIBA have developed and demonstrated some of the ecobased system of farming based on Low Input Sustainable Aquaculture (LISA); like improved traditional system, Organic farming system; including periphyton based farming, brackishwater polyculture system, Bio-floc, and integrated farming system involving rice, fish and horticulture. The ecosystem approach to aquaculture is mainly focused on low input based simple technology suited to the local conditions, providing sustainable, economically viable and socially acceptable models. India has vast resources of traditional farms, which are close to nature, which can be easily modified to suite these technologies. Most innovations and development of brackishwater aquaculture show casing the economic earnings is mainly due to the “industrial” aquaculture, using SPF seeds, formulated extruded feeds, aeration and with the use of various pond management inputs. Balanced growth of these different trajectories on complementary and integrated mode is the need of the hour. Eco based innovative technologies like Biofloc or periphyton will certainly ensure to develop ecologically integrated aquafarming systems that are community-based, sustainable, and economically viable, along the side of industrial farming sector.

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Biofloc and periphyton based farming



Biosecured Probiotic based farming



Organic farming system



BIOSECURITY AND BEST MANAGEMENT PRACTICES IN SHRIMP AQUACULTURE

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Aquaculture has evolved from a simple but an elegant system, which has deep community and family roots. In 1980s, there was a drive towards the export oriented agriculture crops. Thus, many traditional agriculture has grown from basic food producing system to a market driven complex export oriented enterprise. Shrimp culture in the tropics is the paradigmatic example for this transformation. Tropical shrimp farming is considered to be one of the few success stories of modern aquaculture. Evolution of shrimp aquaculture from a fishery based pond production system of 1970s to a mature industry of 1990s is spectacular. Its early success attracted many farmers, and this industry has become the focal point of export in many tropical developing countries. However, the early success and image of risk-free clean-industry has not lasted for many years due to the frequent disease hits and crop failures. Success of shrimp culture often depends on how successfully disease outbreak can be prevented and controlled. Further environmental protection, conservation of biosecurity and social equity are equally important for the long-term sustainability of shrimp farming, although these elements are masked by the short term gains and success.

It is extremely difficult to differentiate suboptimal performance and disease in aquaculture system due to the complexity of this ecosystem. Disease is the end result of series of linked events involving environmental factors, health status of cultured stocks, presence of an infectious agent and poor husbandry. In order to prevent the disease outbreak and negative environmental effect of aquaculture, the whole aquatic ecosystem including ecological process must be taken into consideration. The traditional pathogen-focused approach, therefore, should be replaced by more holistic approach focusing the whole ecosystem. The best management practices and the strict biosecurity measures are the essential tool to manage the disease and environmental health

History of best management practices can be traced back to the history of aquaculture or the history of any production system. It is evolved from the producers' quest to reduce the input and costs, and vast majority of the best management practices are generated by the producers. No single BMP reduces key impact equally, as there is no one-size fit for all. The most effective BMP depends on species cultured, type and magnitude of impact, scale of production, resource available to producers and overall management of the system. As best management practices in aquaculture and biosecurity protocols are intimately linked, in this lecture note, these aspects are treated together.

Biosecurity

The entire stake holders of aquaculture concerned about biosecurity: Consumers need to ensure the seafood that they eat are safe, the processors have to follow HACCP guidelines to provide safe seafood, investors should protect their investment from the preventable losses. The Biosecurity workshop for aquaculture defined biosecurity as: “an essential group of tools for the prevention, control, and eradication of infectious disease and the preservation of human, animal, and environmental health” The principles of bio-security are not only to keep away the pathogen from the farming environment but also from the country.

The success of poultry industry world-wide is the successful implementation of biosecurity. It has been prompted the use of similar protocol in shrimp farming. In Poultry biosecurity is defined as: “cumulative steps taken to keep disease from a farm and to prevent the transmission of disease within an infected farm to neighboring farms.” It is a team effort, shared responsibility and an ever-time process. Basic philosophy behind biosecurity is to prevent the entry of pathogen, ensure the best living condition to the animals and to provide a clean product to the customer. The principles of biosecurity in the poultry can be applied to the aquaculture

Strategies	Benefits
Sludge removal and disposal away from the pond sites	Increase the carrying capacity of the pond, and improve the pond general conditions
Adoption of minimal water exchange	Increase the stability of culture environment, minimize the entry of influent pathogens
Water filtration using twin bag filters of 300 μm filters	Prevent the entry of disease carrying vectors
Water treatment using approved chemicals such as chlorine, and aging the water	Eradication of pathogens
Maintaining the water depth at least 80 cm at shallow part of depth	Prevent the formation of benthic algae

Site selection: Poorly located sites are often found to be failed and provide negative ecological impacts. Potential problems should be identified and measures should be taken to avoid maximum problems. Mangrove sites and other coastal wet lands should be avoided, as these habitats are inherently important for ecological well-being.

Farm design: Modular seawater system with reservoir ponds before use in culture is found to be effective. All these farm design directly depends on the characteristic site and level of intensity.

All inlet and outlet system should be free from leakage, and to avoid carriers such as crabs and birds, preventing nets should be installed. Additionally, the management measures to improve soil quality and other preventive measures should be taken as per the following Table

Broodstock and post larvae

During the early days of shrimp farming, farmers used wild seed stock entering along with the tidal inflow or captured wild broodstock. This practice was replaced later with the use of hatchery produced seeds obtained from the wild caught broodstock. This wild caught broodstock are often carriers of pathogens. Thus, it is understood that dependence of wild broodstock are important source of pathogen entry, and without de linking the wild fishery and aquaculture, the disease management cannot be attained effectively. Thus, use of captive reared and specific pathogen free broodstock are found to be crucial. The process of development of specific pathogen free broodstock are given below

Development of Specific Pathogen Free stock

Whatever the methods have been incorporated to eradicate the occurrence and out - break of disease in aquaculture ponds, none could provide enough protection, if we use seed stocks derived from the wild brooders. Therefore, the most important principle of biosecurity is the use of domesticated stocks, which have been cultured under controlled conditions and that have been under active disease surveillance program. The development and use of specific pathogen free (SPF) stock is, perhaps, the best management strategy for stock control in farms or regions or countries. Although in market place, these stocks are called as “disease free” in reality they are free of specific disease causing agents. SPF means the stock of interest has at least 2 years of documented historical freedom of pathogens listed on the working list. This pathogen should have the following criteria: 1) the pathogens must be excludable; 2) adequate diagnostic methods should available and 3) pathogen should pose significant threat to industry.

The process of SPF development begins with identification of wild or cultured shrimp stocks. The samples of this stock then will be tested for specific pathogens using appropriate diagnostic procedures. If these stocks are free from specific pathogens they are designated as founder population or F_0 , and they will be reared in a primary quarantine facility. During the primary quarantine F_0 stock will be monitored periodically for the specific pathogens. If this stock is detected for any of the specific pathogens, the stock will be destroyed. The stock will be moved to secondary quarantine, if they are free of specific pathogens. At this facility these stocks will be matured, selected and produce F_1 generation. These F_1 stocks

will be maintained in quarantine further to ensure that they are free from specific pathogens. These SPF stocks will be supplied to hatcheries and breeding centers.

Use of stress test

Exposure to weak concentration of formalin or with change in salinity can be used to determine whether post larvae are strong enough to survive stocking into ponds.

Feeds and feed management

Manufactured feeds account for 60 -70% of total operating cost in shrimp aquaculture. Feeds are one of the important concerns for environmental group because it depends marine capture fishery for fish meal and fish oil. Further, 20 to 40% of feed becomes remained unused by shrimp become pollutant to the pond. Use high quality feed, and feed should not contain more nitrogen and phosphorus than shrimp needed. Feed management practices should be carefully monitored. It should be assured that shrimp consume as much as feed shrimp consume, to avoid the wastage of feed. Check tray should be used to avoid over feeding and under feeding. Feeding should be practiced four to five times per day, and it should be broadcasted widespread. Feed should be adjusted with biomass and appetite of shrimp. Natural productivity has an important role in the nutrition of farmed shrimp. The larvae at early stages cannot consume pellet feed as efficiently as larger shrimps, and therefore, natural biota of the culture pond plays an important role in the nutrition during the early phase of culture. Therefore, production and maintenance of natural productivity has important role in the sustainable shrimp farming. Do not use fresh feed or other material for feeding the farmed shrimp.

Health management

Regular monitoring of shrimp for the health status should be carried out, the sick and moribund shrimp should be removed regularly. In the case of disease out-break of disease strict quarantine protocol should be followed to prevent the spread of disease. As many stressors reduce the innate immunity of many cultured shrimp, the measures should be taken to minimize the stress such as maintenance of high oxygen content in the water, maintaining stable pH, temperature and salinity of rearing water, minimize the use of feed, water exchange etc. The eradication of disease at the beginning is easier and do not use antibiotic. Use probiotics judiciously and only when the efficacy of the product is proved.

Conclusion

BMP and implementation is most crucial component of successful shrimp aquaculture. The most important step of the biosecurity is the exclusion of pathogen from the system. Education on biosecurity makes farmers more awareness. It provides set of tools to protect the crop, and eventually it makes shrimp farming more profitable and sustainable.

INTRODUCTION TO BIOFLOC TECHNOLOGY: PRINCIPLES, PROSPECTS AND CHALLENGES

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Introduction

Biofloc based farming is becoming popular and can achieve higher productivity through sustainability befitting to the sustainable intensification concept. This technology is based on management through heterotrophic microflora promoted and maintained through C:N ratio manipulation (Yorum, 1996; Tsamocha et al., 2009; Panigrahi et al., 2020). It is based on retaining waste and its conversion to microbial floc as a natural food within the cultural system. Science-based BFT farming is promoted by optimizing inputs, protein content in the feed, C: N ratio, and floc microbial constitutions. Maximizing productivity from minimum natural resources is recognized as one of the most vibrant futuristic technology under the Govt of India (PMMSY) Scheme. It is practiced in both freshwater and brackish water system with a wide range of species like shrimp (*P. vannamei*, *P. indicus*, and *P. monodon*), Tilapia, Pungas, Amur & other carps, native air-breathing fishes such as singhi, koi, and magur are also reported to have better growth performance in tank based farming system.

Bio-floc is an assemblage of beneficial microorganisms such as heterotrophic bacteria, algae (dinoflagellates & diatoms), fungi, ciliates, flagellates, rotifers, nematodes, metazoans & detritus. As it contains predominantly heterotrophic bacterial community over autotrophic and denitrifying bacteria, this can be controlled by maintaining high carbon to nitrogen ratio (C:N). Biofloc in combination with periphyton (BPT) increases the natural production and in turn productivity. The lower/minimal water exchange facility in biofloc system also improves the pathogen exclusion in culture pond.

BFT supports high density and biosecurity, maintain the waterquality even in the absence of water exchange, maximum utilization of nitrogen input in the form of feed and finally results in economically viable system. Through developing dense heterotrophic bacterial community by C:N ratio manipulation, the system becomes bacterial dominated rather than algae, and maintains water quality through *in situ* bioremediation. As disease outbreaks and their impact on commercial shrimp farming operations during the past two decades greatly affected the operational management of shrimp farms, BFT approach promises a healthy rearing system, which is increasingly identified as one possible approach for disease prevention.

Principle

The fundamental principle of biofloc is the nitrogenous waste can be converted into microbial biomass under strong aeration, those floc protein food which could provide an adequate amount of food source for shrimp culture. In a typical brackishwater pond, only

20–25% of fed protein is utilized by the fish/shrimp, rest of which goes as waste in the form of nitrogenous metabolites. Manipulation of carbon: nitrogen ratio in shrimp/fish ponds encourages the uptake of this inorganic nitrogen into microbial protein known as biofloc. 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 12-15:1) by adding different locally available cheap carbon sources and / or reducing protein percentage in feed. Under optimum C:N ratio, inorganic nitrogen is immobilized into bacterial cell while organic substrates are metabolized.

<p align="center">Heterotrophic system</p> <p><i>Requirements:</i> [ammonia] + [oxygen] + ["alkalinity"] + [organic carbon]</p> <p><i>Products:</i> [bacterial biomass] + [water] + [carbon dioxide]</p>
<p align="center">Autotrophic system</p> <p><i>Requirements:</i> [ammonia] + [oxygen] + ["alkalinity"]</p> <p><i>Products:</i> [bacterial biomass] + [water] + [carbon dioxide] + [nitrate]</p>
<p align="center">Mixotrophic System</p> <p><i>Requirements:</i> [ammonia] + [oxygen] + ["alkalinity"] + [organic C from feed]</p> <p><i>Products:</i> [bacterial biomass] + [water] + [carbon dioxide] + [nitrate]</p>

Different types of aquaculture systems

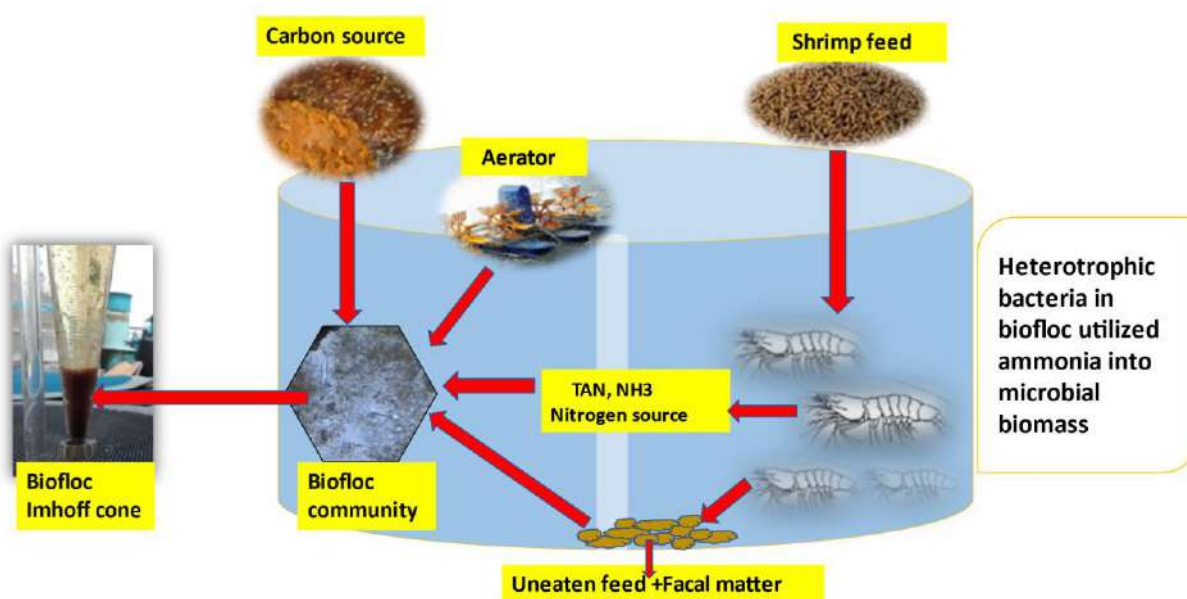


Fig. Schematic diagram showing the concept of biofloc in a grow-out system and b) Nitrogen conversion pathway in biofloc system.

Evolution of biofloc concept

As early as in the year 1976, Steve Surfling put forward the 'microbial soup' concept that eventually led to the development of "bio-floc" based aqua farming. Dense heterotrophic bacterial community is developed to make the system bacterial dominated. Accumulation of these bacteria, called flocs, engulf up the nitrogenous wastes ten to hundred times more efficiently than algae, and turn them into high-protein feed. In early 1980s BFT was developed at Ifremer-COP (French Research Institute for Exploitation of the Sea, Oceanic Center of Pacific) with different penaeid species including *Penaeus monodon*, *Litopenaeus vannamei*. Later, Prof. Yoram from Israel, contributed immensely for the further modification and promotion of this encouraging technology. The technique developed in Israel subsequently spread to many other countries due to its several advantages.

Microbial community and their management

At elevated C:N ratio, bacterial bio-floc recycles nitrogen to keep TAN below safe level (1ppm) enabling shrimps to be stress free in pond ecosystem. Based on the contributing microorganisms, three basic types, green, black and brown bio-floc systems have been categorized. Brown flocs are more heterotrophic and gave better stability to pond, nutritionally better and more predictable. Also the bacterial biomass need to be in suspension through constant aeration of 35-50 hp/ha. The number of total bacteria in bio-floc group was significantly higher (10^6 to 10^8 cfu mL⁻¹) than that of conventional system. The "natural probiotic" effect in bio-floc could act internally and/or externally against, *Vibrio* sp. and ectoparasites. While Jang (2013) recorded 351-773 operational taxonomic units (essentially equivalent to "species") in water from bio-floc systems many as thousand which could be there in such a conglomeration and proper environment and management strategy should be there to optimize the system.

Advantages of bio-floc based aquaculture technology

- Biosecurity of the system can be maintained with Zero/minimal water exchange system
- Heterotrophic bacteria can reduce toxic metabolites (NH₃-N, NO₂-N)
- Easier management and environmental friendly approach (reduced protein requirement, fish meal usage and water/nutrient discharge), diurnal changes (pH, O₂, CO₂) in pond water is reduced
- Doubling the protein utilization as the shrimp use proteins twice - eat feed and then harvest flocs. Enhance digestion (with enzymes and growth promoters)
- Probiotic action - more diverse aerobic gut flora reducing pathogenic bacteria (*Vibrios*).
- Role in immune response by stimulating humoral and cellular immunity
- Reduced costs (15-20% lower cost of production) including 30-50% cost savings in feed
- Augmentation of natural food and improvement of FCR
- Reduced sensitivity to light fluctuation

- Major advantage of growing shrimp in biofloc will not require of multiple external filtration. It reduces the start up operational expenses.

Limitations

- To sustain the bio-floc, high stocking density-biomass of shrimp is required.
- Since oxygen is very critical, aeration and energy cost increases
- Involved more technicality and understanding of the system
- Start up time period required
- Limited progress due to lack of proper technical and infrastructural facilities and restrictions

Bio-floc based integrated nursery and grow-out system

Nursery rearing of penaeid shrimps enhances the growth and survival of shrimps in grow out systems. BFT has been applied successfully in nursery phase in different shrimp species such as *L. vannamei*, *P. monodon*, and *F. setiferus*. Bio-floc and periphyton based nursery systems results in increases of 30 to 50% in weight and almost 60-80% in final biomass in shrimp at early post larval stage when compared to conventional clear-water system. Other advantages include better health and increased immunity through the continuous consumption of bio-floc which in turn positively influence grow-out performance. In a trial in India, the weight of the shrimp postlarvae/juveniles were enhanced from 15 to 250mg with rearing densities above 10,000/m³ of water showing better performance in bio- floc system for *P. monodon* and *F. indicus*. Nursery rearing under bio-floc system gives better result in terms of performance (growth and survival) and protective response.

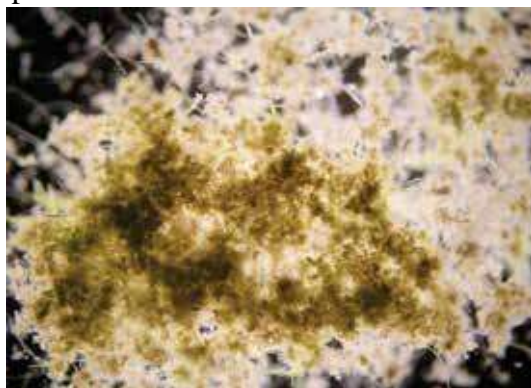


Fig: Biofloc image taken under phase contrast microscope (4X). Fig. Nursery reared shrimp

Recirculating shrimp nursery systems with the use of bio-floc shows promising results and reduces the size and cost of the filtration system. It is also reported that the bio-floc also can reduce the costs for standard starter feed up to 50% without compromising the growth, health and survival of the animals. The bio-floc based nursery can improve the optimization of farm facility with high stocking density in nursery phase along with ensuring the successful cross over from early mortalities. Several studies indicates that bio-floc with

periphyton systems (BPT) increased growth, survival and protective response further while also contributing to more favorable water quality.

Similar advantage in bio-floc based grow-out systems has also been reported by many studies. As 20-30 % of the shrimp feeding is taken care by the floc particles, there is a potential gain in FCR. The selective breeding program for Pacific white shrimp, *L. vannamei*, involving the super-intensive shrimp culture with bio-floc has been conducted at Oceanic Institute in Waimanalo, Hawaii, USA, since 1997. These trials are conducted in a 75-m³ super-intensive BFT raceway stocked at 300-400 shrimp/ m³ in Oceanic Institute's Nucleus Breeding Center. In an experimental microbial floc culture system, shrimp given feed with less than 25% crude protein performed similarly to shrimp raised under regular intensive culture with a 37%-protein diet.

Reproductive performance under Bio-floc

As closed-life cycle broodstock shrimp and captive breeding with genetic improvement program become priority. Biofloc model with its integral biosecurity can be successfully adopted as it also assures stabilized water quality parameters in indoor facilities and healthy shrimps. Improved spawning performance of *L. stylirostris* and *F. duorarum* shrimps is reported in BFT systems. The high protein-lipid rich nutrients in bio-floc, including fatty acids, vitamins, phospholipids could be utilized continuously and there by help in building reserve energy, broodstocks gonads formation and superior reproductive performance.

Bio-floc and Periphyton based research and development

Considering the feed composition, immune effects, shrimp growth rates and other properties, development of target oriented bio-flocs are being widely practiced in academic institutions as well as by commercial companies. The regular addition of carbon to the water is known to select for polyhydroxyalkanoates (PHA) accumulating bacteria which produce several biodegradable polymer storage products, like poly-β-hydroxybutyrate (PHB). The cell wall components of these beneficial microbes have potential immunomodulatory properties. Studies in the similar line also revealed an up- regulation of immune genes with exposure to bio-floc implying immunomodulation in the shrimp.

In India institutes like CIBA and TNFU have recognized the potential of this technology and its environmental benefits (which otherwise proved a major impediment in nutrient dense production system) and projects are going on to further standardizing a set pattern; so that the farmers can adopt this in nursery and grow-out production practices. Among the various studies reported worldwide, a higher production of 20 to 50 tons per ha was obtained with this technology. In India, also a production of 16-18 tons of *P. monodon* and 30-45 tonnes of *L. vannamei* has been reported using the biofloc technique where carbon addition was practiced with very high aeration rate. Hi tide sea farm at Tamilnadu have pioneered the bio-floc and periphyton based farming in India. Similarly, in few entrepreneurial farmers in Andhra Pradesh are getting into the BFT system of shrimp farming. While there are instances of very healthy harvest, certain experience of failure have also been recorded which needs further investigation and standardization. Even in low

input based farming systems, addition of molasses or other yeast derivatives is being added by farmers to have good bacterial population and stable plankton bloom.

In our experimental trial at ICAR-CIBA, application of bifloc technology in white shrimp *F. indicus* and pacific white shrimps by modulating the C:N ratio and at different protein levels have shown better performance in terms of growth and survival of shrimp. Further, there was substantial gain in the production as well as FCR in tiger shrimp *P. monodon* farming by following these eco-based periphyton techniques. At ICAR-CIBA we have successfully demonstrated the bio-floc and periphyton based nursery technology for shrimp culture in nursery tank system, where a very high survival of 98-99% was achieved compared to the conventional system (91-92%).

Biofloc improves production performance

Through adoption of biofloc based system, total biomass can be escalated up to 41% when biofloc concentration was managed through the use of external settling chambers compared to conventional farming. Also, the yield can be further increased if RAS systems are incorporated with floc systems. Recently our protein sparing experiment, a production level of 4 to 4.5 kg/cu. m (40 to 45 tones per ha) was achieved through this BPT system compared to 2.5 to 3kg/cum in conventional autotrophic system (Panigrahi *et. al*,2014).



Fig: (A) Bio-floc cones (B) Shrimps harvested in BPT (C) Periphyton study

Bioflocs on *in situ* bioremediation

As it is well documented that amount of organic matter, nitrogen, and phosphorous discharged in the effluents range from 500 to 1625 kg, 26 to 117 kg, and 13 to 38 kg, respectively, for each ton of shrimp harvest, bioremediation through microbial floc can play a pivot role in maintain environmental sustainability.

Biofloc as Immunomodulation and disease control

- Biofloc have the potential to provide shrimp with pattern recognition and other molecules that lead to stimulation of the non-specific immune system.
- As microbial community develops, a density in the order of 10^7 colonies forming units/ml⁻¹. Beneficial micro-floras enveloped in the system helps to prevent colonization of pathogenic bacteria and improve animal health through the induction of

the immune system.

- Since Biofloc technology (BFT) systems developed to minimize effluent discharge, the system protects the surrounding water resources and improves farm biosecurity. Continuous aeration in the system avoiding stratifications, releases anoxic gases and the stable water creates better stress-free environment.
- Diverse beneficial bacterial community in the biofloc can stimulate the non-specific immunity and limit establishment of pathogenic strains.
- The settled solids and suspended solid waste in this system are recognized and are removed from the biofloc system through central drain system or sludge removal prevents the risk of disease from the sludge.
- Priming of immune system of the host helps in immunomodulation and disease resistance in the animals reared in this system. Gene expression measured in mysis, post larvae and adult of *L. vannamei* was found to be enhanced in the presence of biofloc. Our studies suggest that microbes associated with bioflocs may enhance expression of certain immune-related genes.

Bio-flocs as biocontrol measure

- Bio-flocs can act as a natural probiotic, which could act internally and/or externally against, *Vibrio* sp. and ectoparasites.
- Regular additions of carbon stimulate polyhydroxyalkanoates (PHA) accumulating bacteria that produce several biodegradable polymers like poly- β -hydroxybutyrate (PHB) with biocontrol properties.
- Beneficial bacterial communities in biofloc system controls the pathogenic *Vibrio* population regulates the expression of virulence factor (Quorum sensing) and results in better growth and survival rate attributed to up regulation of immune related genes.

Biofloc as feed for aquaculture species

The nutritional quality of biofloc to cultured animals is good but rather variable. The dry-weight protein content of biofloc ranges from 20 to 50 percent, with most estimates between 25 and 45 percent. Fat content ranges from 0.5 to 15 percent, with most estimates between 1 and 5 percent. Being a rich source of essential nutrients like aminoacids, fatty acids, vitamins biofloc forms an ideal feed ingredient or dietary supplements. Studies reveals that externally produced biofloc can be used as a fishmeal replacement purpose.

Researchable issues and challenges

- Customized biofloc preparations (with inoculums of defined probiotics and algae), modulating the bio-floc yield by C: N ratio manipulation and their evaluation for host performance is a matter of further research.
- Molecular and biochemical characterization of microorganisms (quorum sensing), constituent of biofloc including heterotrophic-autotrophic organism through community approach

- Improvement of floc nutritional value (i.e. using different carbon sources or a mixture of phytoplankton and bacteria) needs further investigations
- Influence of bio-flocs (poly-beta-hydroxybutyrate, Short Chain Fatty Acid –SCFA like constituents) on the protective response in host using immunological and functional genomics-based approaches. Immune response of host by looking at humoral, cellular and immune gene expression at molecular levels when treated after bio-floc/probiotic treatment.
- Exploring the potential antagonistic properties (through *in vitro* and *in vivo* studies), Efficacy of bioflocs to influence viral and bacterial load and its clearance ability should be looked into through pathogenic *vibrio* (immersion or oral) or WSSV challenge studies.
- Elucidating the mode of action of the microbial interventions through bioremedial and ecological approach.
- Evaluating biofloc and/or periphyton based system for advantage. Standardization of the protocol to generate biofloc through manipulation of C: N ratio and inoculums.
- Nutritional characterization of different types of bio-floc and optimization of their inclusion in feeds. Scaling up for bio-floc and mass scale production for further application as feed ingredient.

Strategy to mitigate the challenges

- Development of biofloc within various aquaculture practices—semi-intensive/super-intensive/RAS.
- Focusing more on a semi-floc mode of production system to suite Indian farmers.
- Mechanism to control the unregulated growth of biofloc in the existing pond systems and evaluating biofloc and/or periphyton based waste for utilisation as feed ingredient.
- Extending the BFT/ BPT research outputs on other penaeid species for better understanding, since endogenous species show greater adaptation to local conditions and may be used in restocking programs or cultured in their natural environment.
- Promoting the BFT/BPT based nursery rearing to overcome the disease problem at early stages.
- Working towards shift in policy for these environment friendly technologies with modifications in stocking density or biomass limit and restrictions on use of molasses as carbon source.
- Encouragement of this ecologically sustainable symbiotic system through demonstration and capital subsidy and road map for the development should be prepared.

Conclusion

BFT like eco-based technologies will enable aquaculture practices towards an environmental friendly approach. With the appearance of emerging disease problems and

escalating costs for energy, Biofloc technology can be an innovative strategy for disease prevention and control in contrast to usual tactics such as antibiotic, antifungal, probiotic and prebiotic application. bio-floc technology with biosecure modular systems may be an answer for more efficient, sustainable, profitable aquaculture production. This technology have the obvious advantage of minimizing water requirement, recycling *in situ* nutrients and organic matter and in turn improving farm biosecurity by exclusion of pathogens, augmentation of natural food and improvement of FCR, providing stress-free environment. Use of chemicals and other medicines could easily be avoided thereby having negligible environmental impact. Availability of natural food in the form of microbial bio-floc compensate for higher protein requirement of aquatic species. Biofloc technologies have potential to revolutionize the aquaculture sector. However, this is still in an initial stage and lots of research is necessary for its modification, standardization and implementation.

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BIOFLOC TECHNOLOGY: STANDARD OPERATING PROCEDURE

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Introduction

The aquaculture industry in the world is gaining momentum and is displaying a steady growth in the past two decades. Aquaculture is considered a dynamic food-producing sector, which produce high value protein. Fish is a sought after protein source for not only humans but also for animals, due to its high nutritive composition compared to other protein sources. Above all these features of aquaculture, it was always in the debate regarding sustainability and environmental pollution. In any given aquaculture venture 50% of the cost incurs towards the use of feed. The dependence of aquaculture feed production on the fishmeal is a hot topic in the industry. The search for closed aquaculture system with the concept of recycling sprouts from this thought. These thoughts have resulted in thinking out of the box and producing commercially viable technologies like biofloc and circulatory aquaculture system.

Harboring biofloc in shrimp culture systems to recycle organic and inorganic nutrients by utilizing the services of microalgae and bacteria and providing biofloc itself as a natural feed for the shrimps is a novel concept that is eco-friendly, cost-effective and has considerable potential for further intensification by increasing the shrimp stocking density in a unit area provided. Biofloc based shrimp farming has a great opportunity to fine tune for better production. Biofloc technology can be further improved by better biosecurity, fine-tuning the stocking density, physical, management of water quality. Feed management and carbon addition strategies are the major two corner stones in biofloc technology and this aspect should be handled with at most care for maximizing the profit. In this article, the biofloc based culture system is discussed for adoption of better shrimp culture practices.

Pond design and construction for biofloc based culture

HDPE lined pond with well-prepared dyke is preferred for the biofloc based shrimp farming. In low-lying areas near the creek, the elevation of the pond should be higher than the high tide line to avoid water accumulating under the pond lining. HDPE lined ponds with sufficient elevation and central drainage can be used for effective biofloc based farming. Central drainage system is a must and it helps in the removal of sludge periodically. However, earthen ponds also have their merits as far as interaction with soil phase and effective nutrient release is concerned. Biofloc based system can also be developed in the earthen ponds, but the control over the water quality parameters will be minimal due to soil water interaction.

Bio-floc technology can be conveniently applied in recirculatory aquaculture systems or raceway systems either with *in situ* inclusion or *ex situ* floc production through activated sludge system and putting the harvested bio-flocs in the production system. The system can

be adequately agitated and aerated to keep the microbial floc in suspension.

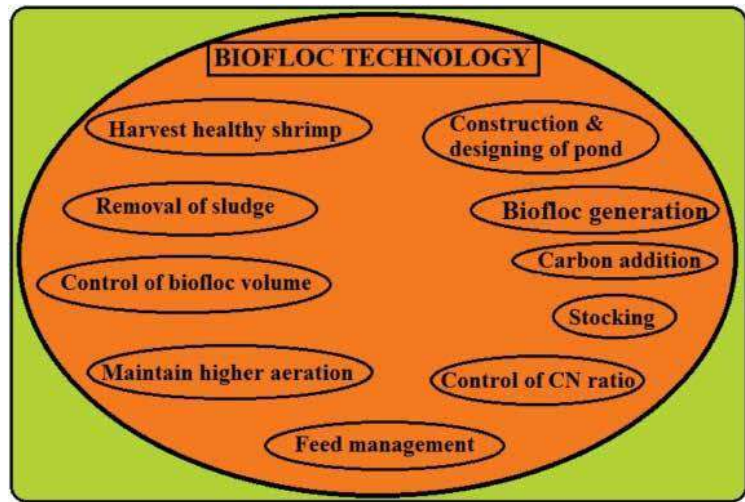
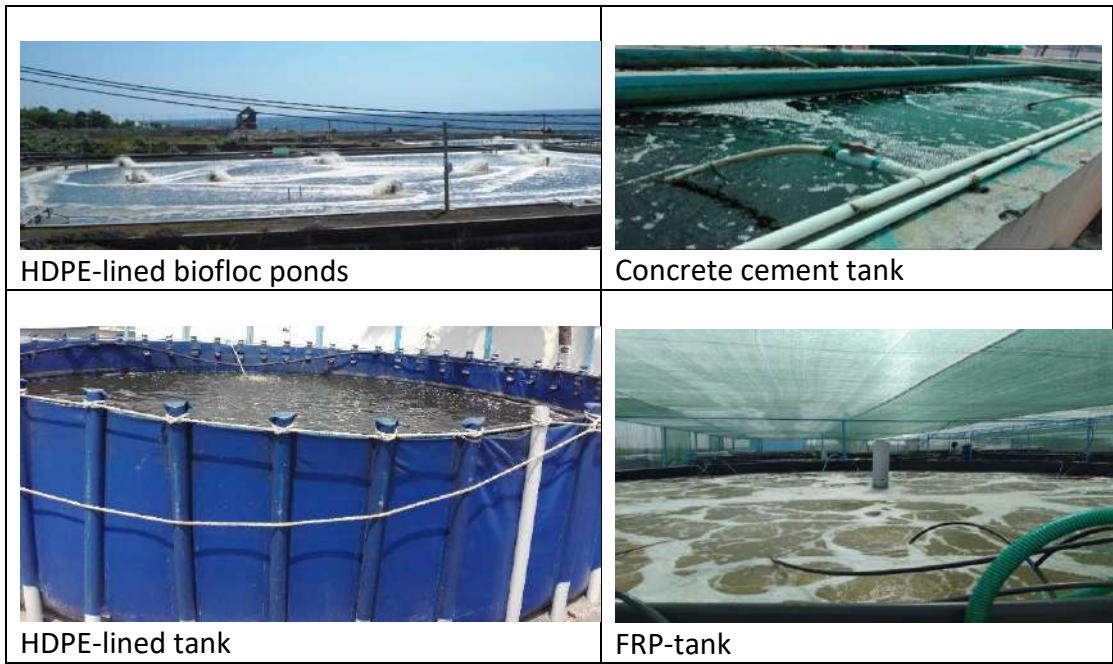


Fig. 1: Schematic diagram of principle and methodology of biofloc technology



Different types of suitable BFT based culture system

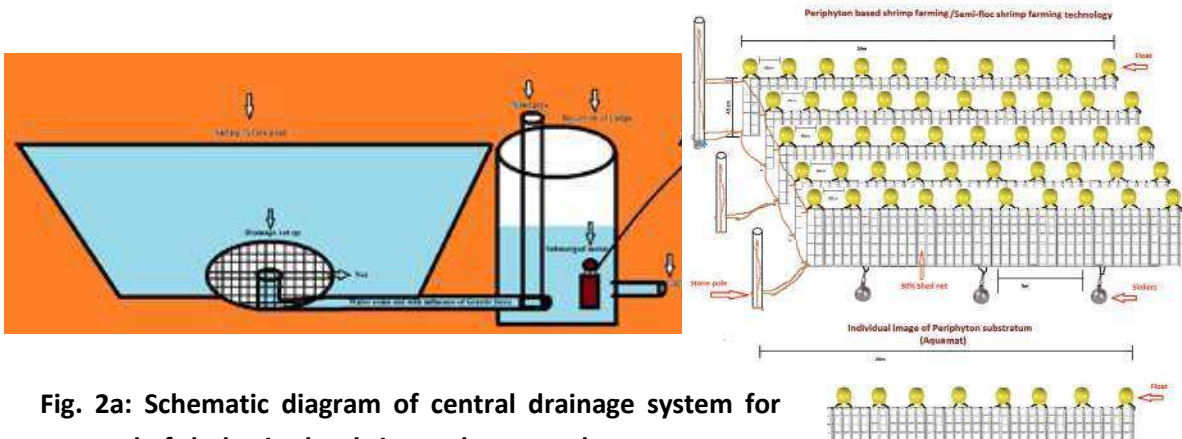


Fig. 2a: Schematic diagram of central drainage system for removal of sludge in the shrimp culture pond

Fig. 2b: substrate for periphyton development in pond

Appropriate animal model and stocking density

BFT technology has been adapted with Tilapia production in aquaculture, which is suitable for high-density culture of species like *P. vannamei* which are effective at utilizing natural productivity in the biofloc based culture system. Other penaeid shrimp species like *P. monodon*, *P. stylirostris* and *Macrobrachium sp.* may not be suitable for the purpose, at high density. Generally, in BFT technology the nursery and grow-out culture of shrimp are segregated. In nursery trial, post larvae with stocking density 5000 to 10000 PLs/m³ and in grow-out culture a stocking density to achieve 10-30 mt / ha shrimp or 1-400 mt/ha fish can be supported. Again, higher stocking under biofloc was found to reduce protein requirement substantially, although still expecting the same growth pattern and better survival to a considerable extent. In contrast, there are reports where biofloc system can be operated with as minimum as 6 nos/m², aiming a higher growth.

Feeding strategies and requirement of feed protein regime in BFT system

Based on the observation of feeding behavior of fish/shrimp in the BFT treatments during the first several days and rationed at 3-1.5% of the total standing biomass daily, feeding rates are to be adjusted depending on the daily consumption and sampling. Daily feed rations are to be split into two to five times to be given at adjusted intervals. Moreover, higher growth was observed when protein levels in the BFT based shrimp culture system were reduced as optimum C:N ratio converts/regenerate microbial flocs/protein (Avnimelech, 1999).

In the BFT system, feed quantity must be reduced in the culture system and feed cost can substantially be reduced by decreasing the protein level in feed. In the case of *L. vannamei*, the growth and survival are reported to be equal or better at 20-30% protein (7% lipid) than 40 % in commercial closed pond systems due to enhanced nutrition on flocs and reduced N-wastes. The guiding standard is that around 95% of N added to pond is from feed/fertilizers and more than 50% of N in feed is released into the environment. One should lower protein content to achieve good C: N ratio and reduce production of N-waste. At <25% feed protein, heterotrophic removal of TAN starts to dominate over autotrophic.

Biofloc generation in the culture system

Biofloc generation is an important phenomenon for shrimp/fish culture in BFT system before or after stocking. There are different approaches to prepare the system for biofloc based shrimp farming depending on the design and compatibility of the system, the species to be cultured, intensity of farming and the buffering time for biofloc generation. Further, the system can operate with complete biofloc or semi biofloc mode with or without integrating substrates for periphyton growth. Ponds are prepared following the Standard Operating Procedures (SOP) like drying, ploughing refilling the ponds with aged soil (minimum for 4-5 days) and disinfected water properly sieved through a 60-100 μ screen and also adhering to all biosecurity protocols. Autotrophic bloom is developed by fertilizing with nitrogen and phosphorous fertilizers and/or in combination with any other biofertilisers. The pH is maintained by liming with dolomite or hydrated lime. In the beginning, nitrogen level is built up using feed or any organic substance.

Subsequently, CHO source like molasses is applied, depending on the feed quantity (while calculating the CHO requirements, the feed CHO content should be taken into account) and the TAN level so that a high C: N ratio (15:1) can be maintained to stimulate the flocs. Typically, ponds start getting dominated by autotrophic algae and after a few days, the water turns brown and foamy as floc develops and the system becomes heterotrophic without much algae. Ammonia levels rises to a peak and thereafter falls, following the rise of nitrite which stabilizes after sometime and can be controlled by adding more C. Transition from algae to bacteria can be stimulated by adding molasses every 1-3 days interval for 2-3 weeks.

Once heterotrophic population establishes, the molasses or other carbohydrate additives can be regulated to keep high C:N ratio based on the TAN level and the feed quantity. There can be a modification in the process if the purpose is to generate biofloc in a shorter duration. If the nursery reared juveniles are stocked, feed quantity is high enough to quick-start the bioflocs with addition of a considerable amount of carbohydrates.

To hasten the process, biofloc inoculum from other ponds or its preserved form can also be applied. However, care should be taken before using any commercial inoculum for this purpose. Heavy aeration is required for keeping the floc under suspension. Pond liner allows easier maintenance of floc in suspension avoiding dead spots and stops accumulation of inorganic material from pond banks/walls caused by excessive water circulation.

i) Natural transition approach

In natural approach we generate autotrophs through addition of fertilizers, feed and other ingredients. These autotrophs have to be converted into heterotrophs by addition of carbon sources and control of C: N ratio maintenance. We can observe the colour transition from green to brown with floc build-up. This method takes a longtime process that means 7 to 10 days for conversion of autotrophic to heterotrophic system.

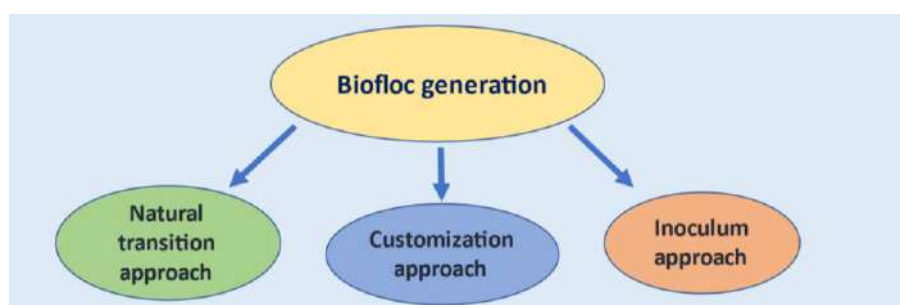


Fig.3: Schematic diagram of various methods to generate biofloc based shrimp culture practices Bio-floc generation may be classified based on the process of making the biofloc with additives in the culture system.

ii) Inoculum approach

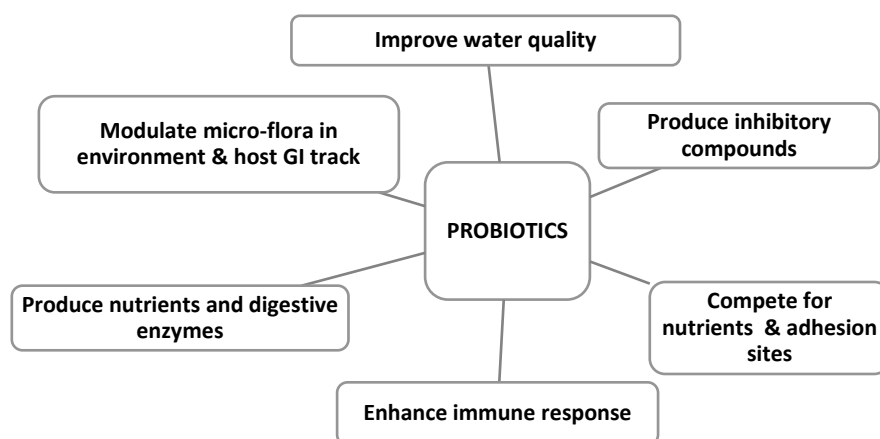
Inoculum approach is based on three aspects i.e. (a) Inoculate the biofloc based culture water of the previous successful crop after checking its suitability in terms of water quality and nutrients. (b) fermented products of carbon sources (molasses, rice bran, wheat flour etc..) and biofloc consortium to be aerated with source water for 24-48 hours and adding the fermented products for biofloc generation (c) cultured biofloc mass produced and dried (fermentation and freeze drier may be employed) to get the powder form. These powders are dissolved and fermented with carbon sources to be added in the form of fermented products. In this approach, biofloc can be generated in a very short duration.



iii) Customization approach

Probiotics are promoted as an alternative health management tool in many fields including in shrimp hatcheries. These natural beneficial bacteria are now well accepted and widely used in fish/shrimp aquaculture practice. Antibiotics not only develop resistant strains creating health hazards, but also are proved to induce immune-suppression. As designated by WHO, antibiotic resistance is a growing public health concern. Different microorganisms (probiotic or microalgae) have been shown to be capable of inducing better production performance and protective response by modulating the immune system in several ways. These probiotics were widely used in culture practices of *P. vannamei* and these strains present in biofloc can be blended in right combinations to get best flocs. Through domestication, the floc can be prepared to contain naturally occurring bacteria that are capable of producing high concentration of enzymes, omega-3 fatty acids having the ability to degrade organic matter and reduce hydrogen sulfide, ammonia, nitrite, and nitrate accumulations.

Probiotics like lactic acid bacteria *Vibrionaceae*, *Pseudomonads* and *Bacillus* are found to give protection to turbot, salmon, cod, shrimp and oysters. The mode of action of probiotics includes the production of inhibitory compounds, competition for chemicals or available energy, competition for adhesion sites, inhibition of virulence gene expression or disruption of quorum sensing, enhancing the immune response, source of macro and/or micronutrients and enzymatic contribution to digestion. Bioremediation involves organic matter mineralization to carbon dioxide, maximizing primary productivity that stimulates shrimp production. Nitrification and denitrification eliminate excess nitrogen from ponds and maintain a diverse and stable pond community by excluding the pathogens from the system by desirable species.



Carbohydrate addition

CHOs are added to promote heterotrophic bacteria (HB) as these bacteria use organic C as energy source and utilize N for their growth. Simple sugars like sucrose and molasses induce the floc to grow faster, however they require frequent additions. In contrast, complex starches i.e. corn, cassava, tapioca, wheat and cellulose are more stable but slow to react and can also act as bacterial substrates and contain suites of enzymes useful for digestion once ingested by shrimp. The lower the feed protein level, the less CHO required. In another study using tilapia farm effluent, it was determined that 1 kg of microbial floc could be produced for every 1.49 kg of sucrose. In minimal/zero exchange intensive systems, excess nutrients are assimilated and mineralized by a dense microbial community in the water column, thus alleviating potential toxicity. (please refer the end of the chapter for C:N ratio calculation)

Management and control mechanism

Floc management is basically done keeping in mind the needs of the bacteria and not the shrimp as at times total bacterial biomass is 2-5 times than that of the shrimp. Floc volumes typically measure 2-4 ml/L first 1-2 months, then 6-20 ml/L later. Total soluble solids should be managed to be less than 300 ppm to reduce aeration requirements. With carbon addition, TAN can be limited at 0.5-1 ppm. The pH should be controlled by addition of lime /dolomite/bicarbonate and better alkalinity should be maintained. Controlling the unregulated growth of biofloc in the pond system is required to avoid a number of critical problems like declining oxygen level and increase in sludge. Hence, proper handling of carbon addition and feeding quantity and schedule as per the standing biomass is required for having a control over biofloc generation.

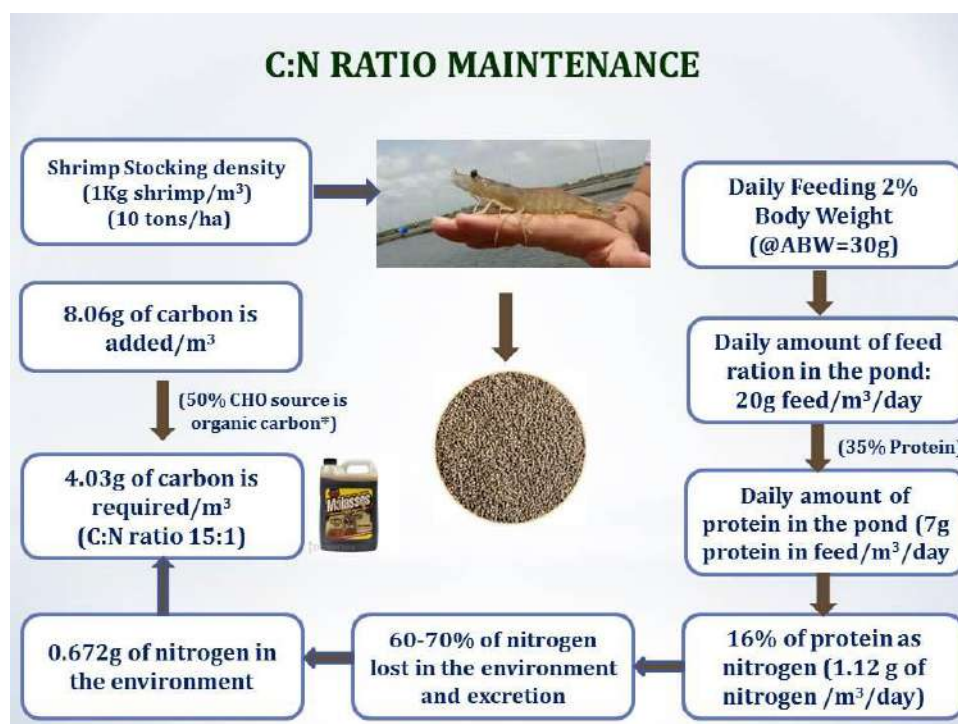


Fig. 4: Schematic diagram of carbon requirements for biofloc based shrimp culture system

Environmental requirements for BFT

BFT works under zero water exchange too, which also makes the system more biosecure. In floc system, ammonia is consumed by bacteria and nitrite increases, with tolerance upto a higher level. There is a need to correct the declining alkalinity which must be maintained >75-150 ppm. Another important requirement in the bio-floc system is to meet the high oxygen demand. There will be also need to suspend the aerator of aspirator type at the sludge pile. Typical aeration requirements are 1 HP/350- 400 kg shrimp biomass. The biofloc system requires high aeration/mixing with aerators and blowers not only to provide DO and remove CO₂ but also to keep biofloc in suspension and prevent production of nitrogenous metabolites, sulphides and organic acids.

Water Quality Management

During the culture period, the water chemistry in the system should be monitored and maintained. Mainly few water quality parameters should be monitored on daily basis other should be monitored periodically like every 3 days once. Dissolved Oxygen, pH, Temperature, turbidity can be monitored twice a day, NH₃, NO₃, NO₂ and TAN can be monitored on daily basis. Algal community, alkalinity, hardness salinity can be seen once a week. Floc volume should be maintained at 12-20 ml/L should be maintained. Total heterotrophic count can be monitored on every 3 days once. The levels of water quality parameters should be kept -in optimal condition for retaining the shrimp's health. All the physico- chemical parameter should be maintained at optimal level for better production of shrimp.

Recommended water quality parameter for biofloc based culture

Parameter	Ideal range	Observation
Temperature (°C)	28-30 °C	Low temperature could affect metabolic rate & growth of microbiota and cultured species.
pH	7-8.5	Less than pH 7.0 could affect the nitrification process
Dissolved Oxygen (ppm)	>5 ppm	For shrimp, fish, microbiota respiration, and growth
Salinity (ppt)	Depends on the culture species	From 0-50 ppt; It is possible to generate biofloc
TAN (ppm)	<1.5 ppm	Toxicity is pH, Temperature, DO dependent
Nitrite-N (ppm)	<2 ppm	Toxicity depends on temperature, DO, Salinity, alkalinity, feed protein level
Nitrate-N (ppm)	0.5-20ppm	Between this range not toxic to the cultured species
Alkalinity (ppm)	100-150 ppm	For help the nitrogen assimilation by heterotrophic bacteria and nitrification process by chemoautotrophic bacteria
Settling Solids (mL L ⁻¹) (Measure in Imhoff cones)	10-15 mL/L for shrimp 25-50 mL/L for Tilapia	Excess of SS and TSS will create oxygen demand, gill occlusion, stress to the cultured species
Total suspended solids (TSS) ppm	250-450 ppm	

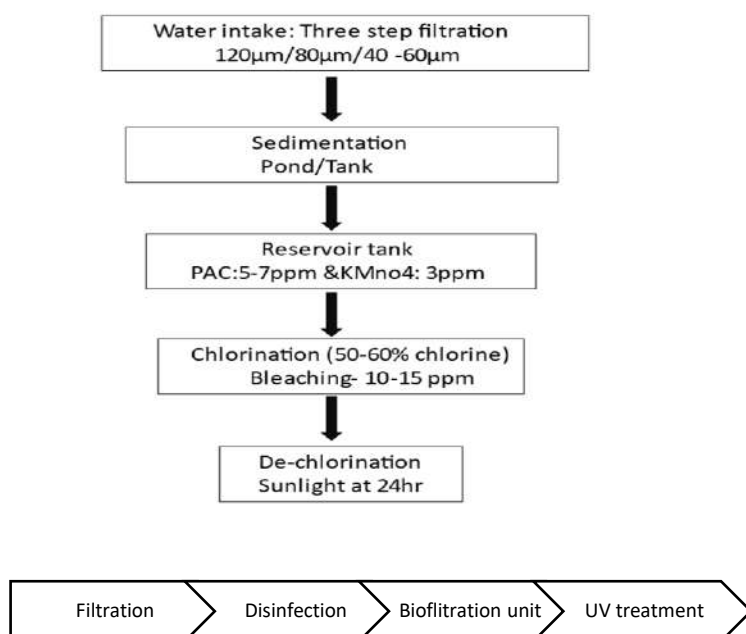
Biosecurity

Quality of Seed: Whether quality of seed is been checked from the vendor on screening of disease and SPF stock availability

Standard practices for seed selection and stocking:

- ✓ Select a registered shrimp hatchery to procure specific pathogen free (SPF) post larvae
- ✓ Select active and healthy shrimp seed uniform in size
- ✓ Shrimp seed should be dark or light brown in colour
- ✓ Seed must be passing a salinity/formalin stress test
- ✓ Test the seed for disease diagnostic in PCR labs, the result should be negative for WSSV, EHP, IMMNV, MBV, HPV
- ✓ Seed bags should have minimum 5L water and enough oxygen (water: oxygen =1:3).
- ✓ Do not pack more than 1000-3000 PL per bag depending on distance. Add Artemia in the seed bag to prevent cannibalism.
- ✓ Seed should be stock at during cool hours of the day i.e. after 8pm and before 8am

Quality of incoming water: This is a more or less controllable variable depending on the rearing system that is applied. The water must then be sampled periodically. This is a key parameter that needs to be monitored and assessed on an ongoing basis.



Disinfection vehicle accessing the farming area: The vehicles entering a farm from feed manufacturers, fish transporter, consultants should be disinfected through tyre bath and spray.

Disinfection of equipment's handled in the pond: Precautionary measures related to pathogens must be applied to all production units - ponds, cages, ponds or aquarium - within each farm. Therefore, the transfers of equipment from pond to pond must be spaced out as much as possible, especially when it comes to the transfers of nets, which are the most likely tools to be directly in contact with animals.

Employee hygiene: farm workers must regularly wash their hands; this rule especially applies to those who manipulate animals through, sorting, feeding, etc. Hydro-alcoholic solutions have proved efficient. In addition, all employees must have their shoes thoroughly decontaminated. In this respect, placing footbaths and brushes at the entrance of each livestock compartment can help limit the spread of pathogens. Keeping a regularly updated register of visits also constitutes an important component of the protection process.

Fencing: Bird fencing and crab fencing must be covering the complete pond for restricting the activity of them.

Conclusion

Standard operation procedures must be followed for sustainable and equally eco-friendly/ biofloc based shrimp culture systems. Not only do they enhance the production, cost effectiveness and prevalence of the disease occurrence is reduced in the vast aquatic environment. Major benefit accrues in the reduced feed cost of the farmers and increased

profit through this technology. Toxic nitrogen metabolites have been prohibited and lead to better management practices of shrimp culture.

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C:N Ratio Dynamics in BFT System and Calculation for its Maintenance

Carbon/ Nitrogen (C/N) ratio in aquaculture production especially biofloc production systems having noteworthy effect of shrimp health and survival. Maintaining proper C/N, would be an important factor followed reducing the ammonia level and this provided a origin for improving development of biofloc aquaculture systems. The maintenance of C/N ratio attributed in two steps: (i) initial development phase, which utilizes a C/N ratio of 12–20:1, and (ii) maintenance phase, utilizing a C/N of 6:1, according to the total ammonia nitrogen values.

Usually enough amount of nitrogen will be available in normal pond system for the growth of heterotrophic bacterial and this can be included mainly by feed. But in Zero water exchange system like biofloc system specifically added with more carbohydrate material mainly having more carbon and less nitrogen. The carbohydrate materials mainly used in worldwide in molasses and apart from this plant based flours, by products like rice bran, wheat bran also now popularly used and it creates the need of nitrogen. Normally in biofloc system C/N ratio maintained above 10:1 ratio level and it pave the way to grow heterotrophic bacterial population.

These bacterial populations take nitrogen for their growth and control the water quality by reducing the ammonia level. Because microorganisms to develop new cells and also for their metabolic activity requires nitrogen and it can be taken up by decomposing organic residues found in biofloc system and It was estimated that 100 grams of organic residue results in 3 to 8 grams of bacterial biomass. Bacteria and other heterotrophic microorganisms have short live spans and divided continuously so that they contribute to

the organic matter pool when they die. Microbial biomass has a low C/N ratio and decomposes easily.

Chemical fertilizers containing nitrogen often are applied in aquaculture ponds offers a quick source of nitrogen to increase the rate of organic decomposition and resulting mineralization of phosphorus to stimulate primary productivity. Another one source is feed and it has narrow C/N ratios (7:1 to 10:1). It was recommended that maintaining higher C/N ratios of 12:1 to 15:1 results in better production in biofloc system, leads to ammonia nitrogen immobilization. Shrimp feed having the crude protein level of 35% and if 6.25 as conversion factor then percentage of nitrogen in feed is more than 5.6%. To balance the nitrogen level external addition of carbon source in pond, in form of sugar, molasses or jaggery which will be utilized along with nitrogen by microorganisms for various metabolic pathways and cell biomass generation. Simply it can be described as the bacteria and other microorganisms will utilize available nitrogen if carbon and oxygen are available to form cell biomass and also for various metabolic pathways.

Before starting the external carbon sources could be fermented with suitable probiotic bacterium will be more helpful to make this method very effective. Fermentation of this carbon sources in sterile seawater for 12-48h with proper aeration makes slurry and after fermentation it could be filtered and applied directly to the tanks. This will increase the count of probiotic bacteria and lowers the chance for *Vibrio* population. Higher density of probiotic applied with proper fermented carbon sources increases the higher rate of waste degradation and enables the clean and healthy pond bottom and water. Dominated increase of good bacteria and planktons gives natural feed for cultured shrimp and reduce the chance for disease.

How to manipulate the C: N ratio?

Before we go into the calculation things to consider is amount of feed used, carbon content of the carbon used to be used, nitrogen amount in feed.

Carbon % in feed

Amount of feed applied	=	1000 gm
Dry matter of the feed	=	90 % dry matter and 10 % moisture
Protein percentage in the feed	=	35 % protein feed (meaning 350gm of protein in 1000gm of feed)
Total dry matter in the feed	=	1kg feed X feed dry matter percentage= 1000x 90%= 900gm

Total uneaten feed by the shrimp is 70%, if so = Total dry matter x % feed unutilized= 900x 70% = 630gm

Total % of carbon in the feed is 50 %, if so, = 630 x 50 % = 315 gm C

C=315

Nitrogen % in feed:

Dry matter of the feed = 90 % dry matter and 10 % moisture

Total uneaten feed by the shrimp is 70%, if so, = Total dry matter x % feed unutilized=
900x 70%= 630gm

= 630 x 35 % protein in feed = 220.5g protein = 33.075

N= 33.075

C/N = 315/33.07= 9.525

C:N = 9.525:1

To make upto 15: 1

33.07 x 15 = 496.05gm carbon source required and feed containing % carbon is 315gm

= Required carbon source - Feed containing carbon source

= 496.05-315= 181.05gm

Finally to make up to 15:1 ratio the carbon source should be added as 181.05gm/kg of feed.

This amount is calculated as per the content of carbon present in various carbon sources

Table: List of commonly used carbon source and their % carbon content.

Carbon source	Percentage of Carbon content (Approx.)	Reference
Molasses	28%	Sujeet Kumar et al., 2017
Sugar	40 %	Serra et al., 2015
Dextrose	40.89%	Serra et al., 2015
Rice flour	40%	Sujeet kumar et al., 2017
Rice bran	43%	Romano et al., 2018
Tapioca	46%	Silva et al., 2017
Jaggery	28.8%	Sakkaravarthi et al., 2015

Example, if we are using molasses as carbon source,

=181.05/28x 100 = 646.6gm of molasses is required to maintain C:N ratio 15:1 for 1kg of feed with 35 % protein content.

=181.05/40x 100 = 452.6g of sugar is required to maintain C:N ratio 15:1 for 1kg of feed with 35 % protein content.

C: N Ratio from different percentage protein feed

(Biofloc Technology: Practical guide. Avnimelech, 2009)

Table: 2 Different percentage protein feed and their C:N ratio

Protein Content %	C/N ratio
15	21.5
20	16.1
25	12.9
30	10.8
35	9.2
40	8.1

C: N ratio calculation based on the TAN level in the ongoing culture

Initially the carbon source applied based on the amount of feed and the protein percentage of the feed applied, later during the culture period, as the floc level increase and available nitrogen source increases due to unutilized feed, excretion etc., and the carbon addition should be based on the TAN level in the culture system. As per the previous reports on C:N ratio, if the TAN level goes beyond 1mg/L, the carbon source addition is recommended with C:N ratio of 6:1

Example

If we have 1.5mg/L TAN was measured in 15000L tank (carbon sourced used rice flour 40% carbon content)

$$= 0.0015 \times 15000$$

$$= 22.5 \text{ gm of TAN}$$

If we want to maintain the C: N ratio with 6:1

$$= 6 \times 22.5 = 135 \text{ g of carbon are required.}$$

$$= 337.5 \text{ gm of rice flour will be required}$$

INTEGRATION OF SUBMERGED SUBSTRATES IN BIOFLOC BASED SYSTEM

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Periphyton can be termed as the matrix of biota, which forms on surfaces, whereas biofloc refer to the matrix of biota in suspension as “flocs. The integration of periphyton in biofloc is gaining importance in aquaculture as it is an eco-friendly and evolving farming practice that utilizes natural food cost-effectively generated within the production systems. Although shrimp feed forms more than 40–60 % of the total operational cost, a major part of the feed remains either unused or excreted as toxic nitrogen metabolites in shrimp ponds, which generally leads to nutrient-rich effluents at the end of harvesting. Optimum harvest of these nutrients and their conversion into protein-rich biomass becomes a major area of interest while exhorting for zero water exchange systems. As we know, biofloc based systems utilize the total ammonia nitrogen generated in the system for the formation of protein-rich flocculated microbial biomass in elevated C: N ratio, which forms an additional quality feed to shrimps. Many times if the amount of floc generated exceeds the shrimp consumption, it can lead to high turbidity in the water column and deposition of particles which demand pumping out the generated flocs. Incorporation of submerged substrates in biofloc system results in the development of microalgal complex over the submerged substrates known as “periphyton” has added advantage in enhancing aquaculture production through improved nutrients utilization and control of toxic nitrogen metabolites. These eco-friendly techniques like periphyton-based farming systems utilize the autotrophic food web or primary productivity as a potential food source for culture fishes. In contrast, biofloc based methods depend on the generation of microbial floc or heterotrophic food web through manipulation of the C: N ratio.

Periphyton-based farming is a widely accepted technology in finfish culture in Asian countries for the fishes like tilapia, Indian major carps, milkfishes, and mullets. Similarly, promising result in terms of growth performance, survival, and production was observed with periphyton in the nursery and grow-out rearing of penaeid shrimps like black tiger shrimps and pacific white shrimps. Periphyton-based aquaculture can be considered a form of organic aquaculture. It provides a consistent form of natural food biota to grazers, apart from being a source of fixed biofilm development, which in turn helps in biofiltration. Both periphyton (fixed film) and biofloc (suspended growth) microorganism cultures are being integrated into diversified farming systems such as RAS, ponds or cages. Being highest in the trophic level, periphyton integration in biofloc provides multiple advantages such as feed substitute grazing on produced biomass with improved Food Conversion Ratio (FCR) and growth rate, destressing, probiotic and pathogen control and, water quality management. As techniques utilize autotrophic or heterotrophic food webs, integration of these techniques makes it



Periphyton on whole bamboo substrate

standalone techniques to enhance productivity. Although biofloc technology has become a highly sought-after technology among shrimp farmers, judicious manipulation of the C: N ratio to maintain optimum biofloc becomes a major challenge. Moreover, high microbial load in the systems demands heavy aeration and pond modification with polythene lining or central drainage systems for periodical removal of unutilized bio floc. At this juncture, the application of immobile submerged substrates can help to trap the suspended microbial floc in the systems. Various researchers found that the use of submerged substrate and C: N ratio manipulation significantly affects the inorganic nitrogen and phosphorous in the water column through uptake of inorganic N ions by bacteria for microbial protein synthesis.

As quality and quantity of periphyton developed on substrates vary with the type of ecosystem, type of substrate, and nutrient availability, manipulation of the C: N ratio can increase the heterotrophic bacterial population over submerged substrates which can be easily grazed by the shrimps than filtering from the water column. When C: N ratio increased from 10 to 20, there were about 40-90 % increases in total heterotrophic bacterial load. Thus, a heterotrophic community facilitated to maintain water quality through the formation of single-cell bacterial protein that acted as a protein source for shrimp, leading to a higher growth rate at a high C: N ratio.

Availability of natural food in the form of epiphytic algae and heterotrophic microbial community compensates for the higher protein requirement of shrimp juveniles and serves as a supplementary nutrient for shrimp. In freshwater systems, increasing the C: N ratio from 10 to 20 increased the net yield of freshwater prawn by 40 %, and the addition of periphyton substrates increased net yield by 23 %. Azim and Little (2006) have reported increased fish production of 30-115% and 30-210% in carp monoculture and polyculture, respectively, and 9t/ha/year in carp polyculture without any supplementary feed. Our experimental trials revealed that integration of submerged substrates improved 18 and 25% improvement in final body weight in substrate integrated C: N ratio manipulated systems compared to biofloc or periphyton-based system alone, respectively. Similarly, a better food conversion ratio, protein efficiency ratio was noticed in integrated systems compared to either C: N ratio system or periphyton system. Since substrates reduce the turbidity by trapping suspended particles, their incorporation in the biofloc system improves the transparency and clogging of respiratory systems of shrimps in the biofloc system.



Shrimps grazing on substrate

Species best suitable for periphyton-based fishery: Aquaculture candidate species with grazing and detritus feeding habits are highly suitable for the periphyton-based fishery. Studies suggest that shrimp are better at grazing periphyton more efficient due to their feeding approach

(Anand et al., 2013). Similarly, herbivorous, detritivorous, and omnivorous fish are claimed to prefer periphyton (Martinez-Cordova et al., 2015). Periphyton-based aquaculture is successfully being practiced for organic tilapia production systems such as *O.niloticus* (Milstein and Omri, 2003), freshwater carps (Azim et al., 2001); freshwater prawns (Asaduzzaman et al., 2010).

What are the commonly used substrates in periphyton integrated biofloc systems?

The type of submerged substrates plays a vital role in the development of microbiota and its biodiversity. Natural or biodegradable substrates such as bamboo, palm leaves, or tree branches, bagasse are traditionally being used among the submerged substrate based farming systems and is found to be effective. The artificial or synthetic substrates are PVC, nylon nets, agricultural shaded nets are also getting momentum and are widely used in biofloc based nurseries or grow-out systems. Recently Seaweed is also gaining importance as a living and potentially edible periphyton substrate, which carries a microbiome of microorganisms that assist in growth and protect the host microalgae (Egan et al., 2012; Singh and Reddy, 2016). Studies evidence variation in quality and quantity of periphyton biomass developed on the type of substrate used with the fast development of periphyton biomass in natural biodegradable substrates due to nutrient leaching ability, although it can add the oxygen demand during nutrient leaching.

Substrate-based fishery helps in nitrification: Autotrophic nitrifying bacteria develop on the substrate and enhance nitrification by keeping ammonia levels low. The dissolved inorganic nutrients such as Nitrate-N, PO₄-P are effectively utilized by the autotrophic community in the periphyton. Thus periphyton act as a biofilter to enhance water quality. Integrated periphyton and biofloc applications provide of multiple genera of bacteria, algae, plankton and simple multi-cellular organisms which can synergistically as an ecosystem and maintain water quality. However, both biofloc and substrate-based biofloc systems demand more DO than the conventional culture systems.

Microbial community in periphyton: Periphyton communities developed in the low light community are dominated by the microbial community, which consists of bacteria, algae, and plankton, and are bonded in a matrix with extracellular polymeric substance (EPS) produced by the (bacteria and algae) over substrates (Pandey et al., 2014). Studies reports, in a 2D fixed periphyton, the biofilm will have inner, intermediate, and outer layers, with each layers dominated by aerobic, facultative and, anaerobic bacteria. This microbial consortia interaction plays a pivotal role in nutrient recycling. When an anaerobic layer in the inner wall generates gases, it results in biofilm slough off unless it is grazed optimally by the culture organisms. However, in living 2D media, e.g., seaweed surface or suspended vegetation root surface will have better beneficial exchanges among the microbial community and interaction environment. The 3D periphyton substrates, apart from enhancing the microbial community, have the potential to upgrade bacterial biomass to a more varied source of live feed, produced in-situ from waste products with biofiltration ability. An example of analysis of the aquatic microbial

diversity in RAS, culturing tilapia with both BFT and periphyton, revealed higher final weight resulted from both types of application but, periphyton demonstrated bacterial consortia carrying lower pathogenic potential(da Silva et al., 2016) with a resilient microbial matrix with the ability to support both nitrification and carbon conversion simultaneously.

Pros and cons of periphyton integrated biofloc systems

Challenges

- Substrates integration needs modification in the design or structure of biofloc tanks or farms with high Energy requirements
- The addition of periphyton to BFT production increases the complexity of the production system and may lead to inconsistencies if not managed properly
- Introduction of substrates can result in alteration of mixing through aeration or diffusion of current flow and can lead to hindrance in the movement of culture species, harvesting, or sampling difficulties
- Substrate integration in biofloc system can further increase the organic load and DO need during night hours

Advantages

- Better water quality maintenance and higher productivity through constant supply of quality natural stable biota
- Better to trap suspended organic load, TSS, and turbidity in high dense biofloc system
- Lower cost substrates input can act as constant sources of both autotrophic and heterotrophic communities can act as an additional supplementary quality feed
- Efficient in the removal of organic phosphate sources from water than biofloc systems
- Biofilm develops over submerged substrates provide a stable nitrification community
- Two or three-dimensional periphyton substrates provide better grazing space for cultures fishes or shrimps and extra living space through generation additional living area. This, in turn, reduces cannibalism and enhances survival during nursery rearing stages
- An organic model with a lower carbon footprint

Conclusion

Periphyton, in combination with a high C: N ratio, facilitates to the maintenance of better water quality by reducing toxic metabolites like TAN, NO₂-N and improving the FCR, growth rate, and survival of the penaeids shrimps. Substrate allows the growth of the periphytic algal community and serves as natural feed, while the increase in C:N ratio converts toxic inorganic nitrogen waste into single-cell microbial protein, which increases heterotrophic bacterial load and lowers inorganic nitrogen content in water. In summary, a high C: N ratio with a substrate system increases the amount and quality of natural food available in the periphyton biomass for shrimp grazing.

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MICROBIAL ROLE IN BIOFLOC SYSTEM

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The primary theme of biofloc technology is to convert nitrogenous waste of aquaculture system into a microbial protein, a valuable nutrient for shrimp and fishes. The biofloc is generated by manipulation of C: N ratio. It serves multitude of function like provision of surplus microbial protein as feed, control toxic ammonia nitrogen and improve the immune system of aquatic animals. The present chapter deals with the various aspects of microbes and the role it plays in biofloc system.

1. Bacterial growth characteristics

Bacteria need simple nitrogen like ammonium ion and carbon source such as sugar, starch, cellulose etc. to run its cellular machinery. This makes it amenable for utilization of toxic ammonia nitrogen from intensive shrimp aquaculture system. Another characteristic feature of bacterial growth is its faster growth rate, as it almost doubles its number within 30 minutes. The bacterial efficiency of nutrient conversion is as high as 50%. Therefore, in nutshell bacteria with its fast multiplication rate are highly efficient in converting toxic product of the aquatic system into the useful, highly nutritious, much demanded microbial protein.

1.1. Carbon nitrogen ratio in bacterial growth

The adjustment of C:N ratio in the feed is the single most crucial factors for microbial growth. This

has been derived by considering;

1. The carbon nitrogen ratio of microbial biomass is around 4.
2. Microbial conversion efficiency range between 40-60% with an average of 50%
3. Carbon content in the feed is roughly 50%

Taking these facts together the carbon nitrogen ratio of 10 has been found optimum for bacterial growth.

1.2 Bacterial nutritional composition

The nutritional composition of bacteria has been presented in Table 1. The table indicates that bacteria have almost 60% protein.

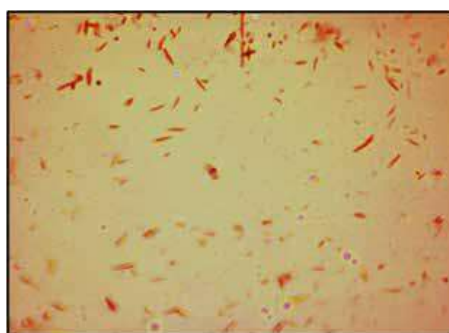
1.3 Aeration is crucial in biofloc formation

Intensive aeration is required in biofloc system to meet enhanced oxygen demand for microbial growth and shrimp. It also assists to keep biofloc in suspension. Our study

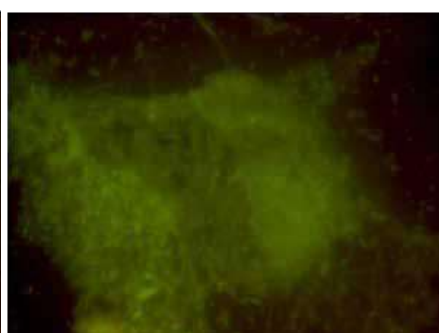
indicated that a drop of oxygen above 1 ppm/h happens in biofloc system compared to control.

Table 1. Nutritional composition of bacteria

Dry matter	25%
On dry matter basis	
Carbon	48.9%
Hydrogen,	5.2%
Oxygen	24.8%
Nitrogen	9.46% (=61% crude protein)
Ash	9.2%



Bacteria in isolation



Bacteria in floc

Fig 1. Microbial constituent in biofloc

2. Microbial system in nitrogen cycle (photoautotrophic, chemo-autotrophic and heterotrophic microbial system)

Three predominant pathways for ammonia-nitrogen assimilation function in aquaculture systems. This includes photoautotrophic, chemo-autotrophic and heterotrophic system. The photoautotrophic system is mediated by algae and diatoms and mostly works at nitrate level, the last and the least toxic metabolite of nitrogen cycle. However, the other two systems (chemo-autotrophic and heterotrophic) system is mediated by bacteria and start functioning from ammonia level, the most important toxic metabolite in shrimp culture. The chemo-autotrophic microbial system is managed by obligate aerobic bacteria and the end product is nitrate. They are ammonia-oxidizing bacteria from the genera: *Nitrosococcus*, *Nitrosomonas*, *Nitrosospira*, *Nitrosovibrio* and *Nitrosolobus*, which catabolize ammonia-N

to nitrite-N and nitrite-oxidizing bacteria from the genera: Nitrospina, Nitrococcus, Nitrobacter and Nitrospira convert nitrite to nitrate. The establishment of nitrification may take between 1 and 2 weeks. The major factors affecting nitrification process in BFT are concentrations of ammonia-N and nitrite-N, carbon to nitrogen ratio, alkalinity, pH, temperature, pH and dissolved oxygen. In contrast, heterotrophic microbial systems not only reduces ammonia level but also convert it into single cell microbial protein, hence serves as foundation of biofloc system. Heterotrophic bacteria are generally classified into floc-forming bacteria and filamentous bacteria. Floc-forming bacteria encourage the aggregation of microorganisms and dead particles to form biofloc and have a small volume but high density. This happens due to production of extracellular polymeric substances (EPS), including polysaccharides that encourages binding of bacterial cells to other particles in the environment. Filamentous bacteria, however, lead to the formation of poorly aggregated bioflocs with a high volume but low density.

However, it is important to note that none of these three systems exists alone in BFT. There is always interplay among these pathways, with the dominant one depending on the management of the system. For example, Xu et al. (2016), noted an algae dominated biofloc system (phototrophic) with C/N 9 and a heterotrophic dominated system at C/N 18. In biofloc the phytoplankton communities mostly falls into the categories of diatoms, chlorophytes, cyanobacteria, dinoflagellates and cryptophytes (Ju et al., 2008). In terms of bacterial community, all the bioflocs had large portions of proteobacteria and bacteroidetes. The research also showed that, bioflocs produced from starch had highest amount of algae, while glucose and glycerol-based bioflocs had well-diversed microbial communities (Wei et al., 2016).

The dominant microbial groups can have some influence on nitrogen removal. Theoretically, heterotrophic dominated BFT would have better performance of nutrient conversion because phytoplankton uptake varies based on daylight exposure. Despite differences in nutrient removal by these three systems in BFT, it is mainly noticed at the initial stage of biofloc establishment and thereafter nutrient removal becomes more stable (Pérez-Fuentes et al. 2016; Kumar et al., 2019). However, most often nitrate remain abundant in systems dominated by nitrifying bacteria or filamentous bacteria (Crab et al. 2012). This is because filamentous bacteria store nitrate-N, which is then released under low dissolved oxygen concentrations (Su et al. 2013).

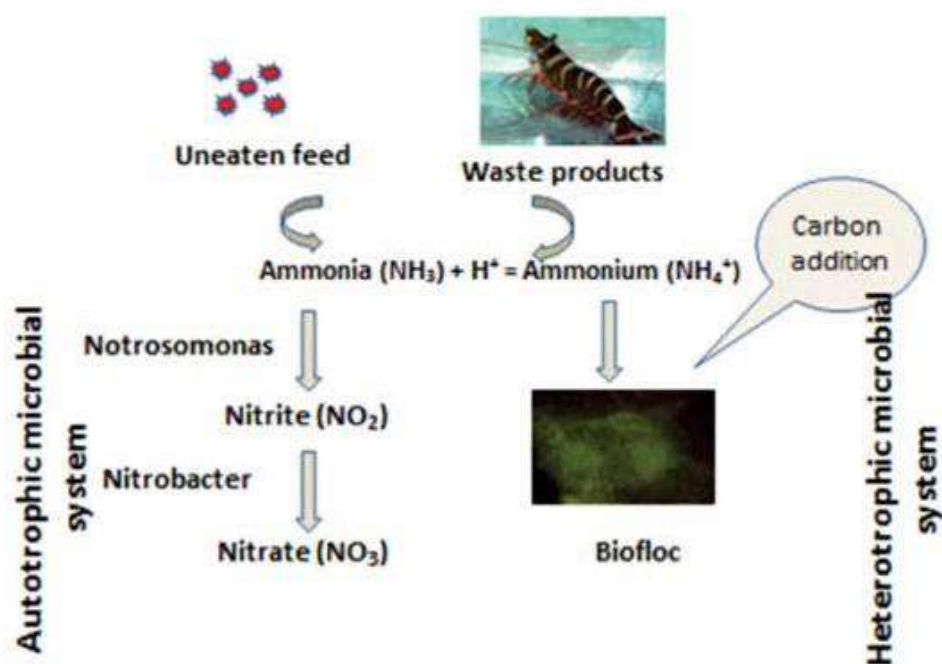


Fig 2. Autotrophic and Heterotrophic microbial system in nitrogen cycle

Table 2. Difference between chemoautotrophic and heterotrophic system

Feature	Chemoautotrophic (Nitrifying bacteria)	Heterotrophic
Multiplication speed	Very slow	Very fast
Generation time of bacteria	Many hours to days	30 min
Oxygen demand	Must, as bacteria are obligate aerobe. Without oxygen these bacteria will die.	Required to keep biofloc in suspension but not indispensable for bacterial growth.
Nitrite accumulation	Chances is high, if aeration is low	

Table 3. Difference between photoautotrophic and heterotrophic system

PROPERTY	BACTERIA CONTROL	ALGAE CONTROL
Energy source	Mostly organic matter	Solar radiation
Occurrence	Dominance in ponds with high supply and concentration of organic substrate, normally limited to intensive ponds with zero or low water exchanges	Ponds with low organic matter concentration. Algae density increases with the availability of nutrients up to limitation of light
Sensitivity toward environmental variables	Does not need light. Adapts to a variety of conditions and stable	Light is essential (activity lowered in cloudy days) Crashes are common and less stable
Effect on Oxygen	Oxygen is consumed and demand is high	Oxygen is produced during the day, consumed at night
Relevant activities	Degradation of organic matter. Production of microbial protein through uptake of inorganic nitrogen	Primary production produces organic matter and oxygen uptake of dissolved nitrate and phosphate
Inorganic nitrogen control	Uptake of nitrogen affected by the C/N ratio of organic matter. Practically unlimited capacity	Uptake driven by primary production
Potential capacity	Limited by substrate concentration and rate of application	Normally, daily primary production $<4\text{gO/m}^2$

3. Microbial consortium of biofloc

The last few years have witnessed extensive research on the microbial composition of biofloc. The recent study suggests that biofloc is mostly dominated by Gram negative

bacteria. Recently researchers reported that most of the screened bacteria belong to Proteobacteria phylum followed by Bacteroides and Cyanobacteria. The member of phylum proteobacteria is widely dispersed in the marine environment and plays an important role in the process of nutrient cycling and the mineralization of organic compounds. It was also found that among Proteobacteria, *Vibrio* group is the most predominant one. Our study indicated that biofloc system had increased level of *Bacillus* and *Lactobacillus* bacterium which have probiotics properties.

4. Microbial role in biofloc system

4.1. Bioremediation of toxic ammonia: The biofloc system maintains adequate water quality especially toxic nitrogen metabolites. At higher C: N ratio, bacteria immobilize toxic ammonia into microbial protein within few hours as compared to slow conventional nitrification process which takes a month to get established. Thus, BFT is essentially a water quality management technique to minimize water exchanges through microbial degradation of nitrogenous toxic waste (ammonia and nitrite) either by conversion to less toxic nitrate or by being converted to microbial biomass.

4.2. Biocontrol agent: Numerous studies have reported that shrimps are healthiest and grow best in aquaculture systems that have high levels of algae, bacteria and other natural microbiota. Probiotics are viable microbial cells and have beneficial effect on health of shrimp by stimulation of immune system and microbial equilibrium in intestine, and by inhibition of pathogenic microbes. Microbes store poly- β -hydroxy butyrate (PHB) as a stored product of carbon and energy. Its synthesis is stimulated in the condition of limited nitrogen supply and with excess carbon supplementation. Condition available in biofloc system thus enhances its production. The PHB particles offer preventive and curative protection in *Artemia* nauplii against luminescent pathogenic *Vibrio campbelli*. This indicates that biofloc can serve as novel strategy for disease management on long term basis. Further, it has been reported that increase in C/N ratio from 5 to 20 reduced the *Vibrio* population (Panigrahi et al., 2018). This suggests the possible role of biofloc system in the control of pathogenic microbes.

4.3. Healthy supplementary food: The protein content of bacteria is almost 60%. Therefore, its consumption becomes an alternate source of protein for aquatic animals like shrimp. The work conducted at CIBA revealed that biofloc is rich in several fatty acids such as palmitic acid (46.54%), cis-Vaccenic acid (15.37%), linoleic acid (10.67%) and oleic acid (9.19%) (Anand et al., 2014).

4.4 Probiotics and immunostimulant: Biofloc is a microbial consortium, which has large number of bacteria, which could play a powerful role in digestive enzyme secretion and as immunostimulant. Our study indicated that biofloc system improves the load of *Bacillus*

and *Lactobacillus* bacterium which is expected to play role in probiotics and immunostimulant effect. Our experimental trial on biofloc work carried at ICAR-CIBA and Kakdwip Research Centre of CIBA, West Bengal revealed that biofloc formation starts 24 hours after addition of carbon source when bacterial count reaches 10^6 - 10^7 cfu. Biofloc was mainly composed of bacterial aggregates, zooplankton and phytoplankton. Our study indicated that biofloc alone or integration of substrate with biofloc system have profound effect on growth performance and immunity improvement in penaeid shrimps (Kumar et al., 2017; 2019). The enhanced immunological parameters such as haemocyte counts, prophenoloxidase, super oxide dismutase, and catalase activity was observed in biofloc groups. Further, the challenge study carried out using pathogenic *V. harveyi* suggested that rice based biofloc system had 55.5% survival compared to only 11% in control (Kumar et al., 2017). Similarly, Panigrahi et al. (2019) reported that biofloc treatment at 32 and 40% protein level significantly increased the transcription of antimicrobial peptides like crustin, MnSOD, hemocyanin, proPhenoloxidase (proPO), peroxinectin (PX) and serine protease (SP).

Several studies are also being carried out using probiotics such as *Bacillus*, *Lactobacillus* sp. in biofloc based system. The data from these studies reflects increase in growth potential and disease resistance abilities against pathogenic Vibrios. Recently, work conducted at ICAR- CIBA evaluated the effect of *Marinilactibacillus piezotolerans* and *Novosphingobium* sp. during the culture of Indian white shrimp, *Penaeus indicus*, under biofloc and clear water systems (Panigrahi et al. 2021). They reported that bioaugmentation by probiotic bacteria significantly improved water quality, especially helpful in ammonia reduction. It also favorably changed the gut microbiota with reduced *Vibrio* population. There was an enhanced expression of digestive enzyme related genes such as trypsin, chymotrypsin, cathepsin L, cathepsin B and alpha amylase and immune genes such as peroxinectin and hemocyanin and antimicrobial peptide crustin.

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DESIGN CONSIDERATION OF BIOFLOC PRODUCTION SYSTEMS – POND, TANKS, RAS AND RACEWAYS

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Introduction

Shrimp aquaculture is one of the fastest growing industries in the world due to an increasing demand for human consumption worldwide. This rapid growth has led to challenges including concerns over environmental pollution, organic loading, disease and parasite outbreaks, and the need to efficiently manage the available resources to maximize productivity. Discharge water management is another important issue haunting the shrimp aquaculture. These factors are advocating conventional production system towards a more efficient management area aimed at the sustainable intensification of the industry. Innovative technologies are being developed and Biofloc production system is one among them as it can improve environmental control over intensive production. In places where water is scarce or land is expensive, more intensive forms of aquaculture must be practiced for cost-effective production and biofloc technology seems to be the solution. Biofloc technology has become a popular in farming of Pacific white shrimp, *P. vannamei* and now it is used in other shrimp and fish as well. It is now a very popular system of semi-intensive and intensive shrimp and fish farming with low or no water exchange. It is comparatively much profitable and bio-secured system of aquaculture.

A basic factor in designing a biofloc system is waste treatment. Biofloc systems work best with species that are able to derive some nutritional benefit from the direct consumption of floc. At its core, biofloc is a waste water treatment system and was developed to mitigate the introduction of diseases into aquaculture facilities or farms from incoming water (water exchange, typically used in prawn farming). Biofloc systems employ a counter-intuitive approach to more traditional aquaculture designs. Where more traditional aquaculture designs seek to remove suspended solids, bio-floc systems allow and encourage solids and the associated microbial communities to accumulate in the water. Assuming sufficient aeration and amalgamation in order to maintain an active “floc” in suspension, water quality can be maintained and controlled. Bioflocs’ may also have probiotic effects in some species. Essentially, bioflocs’ provide two very critical services which also helps a little to understand how they work. They treat wastes from feeding and provide nutrition from floc consumption. It is the utilization of microbial processes within the pond/tank itself to provide food resources for cultured organism while at the same time acts as a water treatment remedy.

Specifications and design of biofloc systems

“Flocs” are a supplementary food resource that can be consumed between feeding times (pellet use). One of the benefits of biofloc systems is its capacity to recycle waste nutrients via microbial protein into fish or prawns. The main component of biofloc is nitrogen that is

incorporated into bacterial cells. One other benefit of biofloc systems is the benefit of improved feed conversion ratios derived from the consumption of microbial protein. It should also be noted that bi-floc systems are generally implemented as pond based systems as they add the most benefits to pond based aquaculture. Additionally, biofloc is not suitable for just any species and works best with species that are able to derive nutritional benefit from the direct consumption of “floc”. Implementing biofloc technology to culture shrimp in ponds and recirculating systems could offer several advantages, including improvement of water quality and animal nutrition.

Basic types of biofloc systems

Few types of biofloc systems have been used in commercial aquaculture or evaluated in research. The two basic types are those that are exposed to natural light and those that are not. Biofloc systems exposed to natural light include outdoor, lined ponds or tanks for the culture of shrimp or tilapia and lined raceways for shrimp culture in greenhouses. A complex mixture of algal and bacterial processes control water quality in such “greenwater” biofloc systems. Most biofloc systems in commercial use are greenwater. However, some biofloc systems (raceways and tanks) have been installed in closed buildings with no exposure to natural light. These systems are operated as “brown-water” biofloc systems, where only bacterial processes control water quality.

Based on the waste treatment, there are two primary biofloc technology systems that can be considered for shrimp culture.

- (i) **in-situ biofloc systems:** where biofloc form in the culture pond/tank along with the shrimp.
- (ii) **ex-situ biofloc systems:** In which effluent waters are diverted into a suspended-growth biological reactor where biofloc are generated and subsequently can be used as an ingredient in shrimp feed.

In-situ systems are currently in use, and ex-situ systems are in the developmental stage. Each option (in-situ versus ex-situ) has unique benefits and limitations. For example, in-situ biofloc systems, under proper conditions, can assimilate ammonia directly into microbial proteins, thereby preventing the accumulation of nitrate (from nitrification). Additional benefits are that the biofloc provide nutrition directly to the shrimp. However, the downside is lack of control regarding manipulation of biofloc nutritional profiles. Furthermore, in-situ biofloc systems exert a high oxygen demand because oxygen is being used by both biofloc and shrimp. With ex situ biofloc systems, one has better control of floc nutritional profiles and can manage the demand for oxygen by floc and shrimp in separate tanks.

Design consideration

Biofloc production systems are either ponds, tanks, raceways or RAS-indoor or outdoor

Pond design-outdoor

Aquaculture ponds are dynamic and complex ecosystems, which will only produce the targeted cultivable production if nutrient cycling and waste decomposition are properly managed. A number of physical, chemical and biological methods used in treating this kind of problem, management practices influencing the load and decomposition rates in ponds include water exchange, sediment removal, aeration, fallowing period between crop cycles, liming etc. Ponds are designed so that it may be possible to maximise the viability of a low water exchange and maintenance of microbial floc system. These specifications include pond shape to maximise active suspension and oxygen distribution, maximum and minimum depths, drainage capability, and lining. The influence of pond design on system viability has not been fully researched and at present different designs are being adopted at different places.

The basic requirements for biofloc system operation include high stocking density and high aeration with correct paddlewheel position in ponds. Ponds must be lined with concrete or high density polyethylene (HDPE). An intensive BFT pond has to be planned bearing in mind the need to provide proper aeration to all parts of the pond, mixing the water to minimize anaerobic sludge accumulation and to enable periodic drainage of the sludge both during the crop and between crops. Additionally, designs should facilitate efficient harvest and easy feeding. General rules and demands of pond design should be followed in designing BFT systems also. But as per the site condition it needs to be modified.

Pond shape

The classical design BFT ponds is based upon a round pond concept with aerators inducing radial water flow, or otherwise square or rectangular ponds where water flow is sort of radial, mostly in parallel to the pond dykes. In such cases, corners are rounded or cut to minimize stagnant areas. Round ponds are the most common design for small ponds, used in hatcheries and some production units. Building larger round ponds is more difficult (digging, utilizing land, lining) and rectangular or similar are more common.

Pond Size

Intensive ponds should not be too large. The biomass in the ponds is high, controlling large volumes of water is difficult, harvesting of too high biomass is complicated and the risk of holding dense fish or shrimp populations in very large reservoirs may be too high. In addition, the risk of losses if something goes wrong in large intensive ponds is very high. The typical size range of intensive ponds is normally in the range of 100-1,000 m² while the typical size of intensive BFT shrimp ponds is 1,000 – 20,000 m² (0.1-2ha).

Pond depth

The depth of ponds is in the range of 1-2 m. The advantage of deep ponds is their high heat buffering capacity, which helps to avoid over-heating or over-cooling during the diurnal cycle. In addition, the deeper water column minimizes contact of the surface water to pond bottom anaerobic conditions and allows a deeper water column for feeding and biological

processes. However, constructing deeper ponds demands a higher investment and, in cases of limited gradient to the drainage base, makes drainage and harvest more of a problem.

Pond lining

BFT ponds are most always lined. It could be lined with Concrete, Bricks, Fiberglass, Wood, Plywood, HDPE, PVC, EPDM. Lining of ponds is usually done with High Density Polyethylene (HDPE) sheets of about 1 mm (30-40 mil). Cheaper alternatives may be constructing a pond bottom with compacted laterite soil (or laterite crushed stones). Laterite, the red soil commonly found in tropical regions, makes a stable pond bottom upon compaction. However, in this case, the banks should also be covered with plastic sheets. Another possibility is to line the pond with a soil cement mixture. In sandy soils one can mix cement with a top layer of soil and obtain a stable lining. The bottom should be smooth to ease draining and cleaning. The relatively fast water movement within the pond (10-30 cm/sec) may induce a significant erosion of earthen banks. Boyd and others (Boyd, 1995) found that such eroded material constitutes a large portion of the accumulated sludge and causes difficulties in pond maintenance. Avnimelech and co-workers (1986) found that a soft clay dominated pond bottom becomes highly anaerobic due to the mixing of organic matter with the clay and the very limited oxygen diffusion into the deep bottom layer. Additional advantages of lining are the ease of cleaning pond bottom in between cycles and possibly more efficient utilization of feed residues sinking to the bottom. It is interesting and important to note that the nature of organic matter accumulating on the pond bottom differs between lined and earthen bottoms. In earthen ponds, the organic residues mix with the soil, forming a rather stable complex, in comparison to highly degradable, unstable and bio-reactive organic residues that accumulate adjacent to the lining. This difference affects pond management: in earthen ponds, organic matter accumulates over a period covering several cycles and has to be periodically removed. In the case of lined ponds, organic deposits do not accumulate, yet due to the high reactivity they affect chemical and biological processes in the pond vigorously and may cause a real problem for production in the pond. One very clear example of the difference between earthen and lined ponds is demonstrated by following phosphorus interactions. In earthen ponds, the soluble phosphorus interacts with soil components and is, to a large extent adsorbed. In lined ponds, such interaction does not take place and excessive phosphorus remains, mostly as soluble phosphorus in the water (Avnimelech and Ritvo, 2003).

Central drainage system

In recent time, there is a noble concept of shrimp toilet or central drain. Aquaculturists are showing interest in establishing shrimp pits or shrimp toilets or central drain at the centre of the culture pond. For this purpose, they are utilizing about 5- 7% of the total surface area of pond. Ideally, the pond size should be about 1000-5000 m² for the establishment of shrimp toilet. Establishments include 7-10 feet concrete cement with a smooth slope to the center where there will be a small well of about 2-3 feet depth. Smooth and slope surface (25-30o) at centre allows fast movement of waste toward the central pit with the additional advantage of lesser requirement of water with concentrated organic waste removal. By the continuous movement of water by intensive aeration all the waste materials will be dragged in to well. This

waste can be removed using a siphoning motor or submersible or floating pump (power of about 2 hp) for every week so that there will not be any sludge. Natural gravitational force can also be used for draining the organic waste like in central drain. In the recent time, there is an addition to shrimp toilet concept is an HDPE and rubber parabola cover (2.5 meters in diameter), placed over the central drain of a pond. The purpose of the keeping parabola is to extend the area of sludge removal. The achievement of thorough drainage is very important in extensive BFT ponds, where a very good exposure to the air and drying are essential. In CIBA, under NFDB project biofloc based *P. vannamei* culture is being undertaken in lined central drainage ponds at Muttukadu experimental station.

Prefabricated ponds/tanks

A new and interesting approach is the installation of pre-fabricated ponds. Such ponds are produced presently in both Mexico and Colombia (possibly else-where as well), are relatively inexpensive and can be installed within a few days. The ponds (tanks) are relatively small (up to about 150 m²) and can be used as a starting technology for individual farmers. Such ponds are suitable for the production of dense fish biomass (shrimp in special cases, to provide fresh shrimp) and can easily be placed in green-houses. Organic residues are always produced and their accumulation as bottom sludge is an unavoidable problem. The basic solution to this problem is to concentrate the sludge in limited points in the pond (sludge traps) and design for the capacity to drain out the sludge, during the production cycle and between cycles.

Race ways – outdoor and indoor

A raceway usually consists of rectangular basins or canals constructed of concrete and equipped with an inlet and outlet. A continuous water flow-through is maintained to provide the required level of water quality, which allows animals to be cultured at higher densities within the raceway. Most raceways are made of reinforced concrete, though some earthen raceways are also built. Earthen raceways with plastic liners cost little and are easy to build, but cleaning and disinfecting them is difficult and plastic linings are fragile. Reinforced concrete is more expensive, but is durable and can be shaped in complex ways. Raceway tanks can also be built from polyester resin. A raceway is most often a rectangular canal with a water current flowing from a supply end to an exit end. The length to width ratio is important in raceways. To prevent the fish stock from swimming in circular movements, which would cause debris to build up in the centre, a length to width ratio of at least six to one is recommended. If the width is too large this could result in a feeble current speed which is not desirable (see below). The length of a raceway unit is usually constrained by the water quality or by how much stock a unit can hold for ease of management. The average depth of a raceway for fin fish, such as rainbow trout, is about one meter. This means each section in a raceway should be about 30 m long and 2.5–3 m wide. The landscape should sloped to one or two percent, so the flow through the system can be maintained by gravity. The raceway should not be curved, so the flow will be uniform. Generally the water should be replaced about every hour. This means a typical raceway section requires a flow rate around 30 liters per second. However, the optimum flow through rate depends on the species, because there are differences in the rates at

which oxygen is consumed and metabolic wastes are produced. The flow rate necessary to maintain water quality can also change through the year, as the temperature changes and the cultured species grow larger. For reason such as these, continuous monitoring of water quality is important, including measurements of water flow rates, pH levels and temperature, as well as the levels of dissolved oxygen, and suspended and solid waste material

Closed raceway

Another BFT system design is the closed raceway approach. The closed raceway is based upon a linear rather than a radial water movement pattern. Closed raceway units can be constructed as such, when all walls and flow partitions are built as an integral part of the system (e.g. walls and partitions built of concrete). A cheaper and easier mode is to put in a partition, dividing the pond the rectangular pond into a closed raceway. It is important to note that the flow partition is separating two sides having the same water head and that the separation does not have to be tight. Thus, a partition made of simple plastic sheets placed in position supported by poles may be sufficient. Aerator placement is generally parallel to the raceways. Sludge tends to accumulate in closed raceways at the ends of the flow partitions where water flows around the baffle and relatively dead volumes are created. The linear flow mode of the closed raceways seems to enable the operation of long ponds. Pond length can be as long as there are enough aerators along the pond (and as long as you can have all pond length sloping toward the outlet). The width of the raceway should be such that enable smooth water flow and easy access. A width of about 10-30 m seems to be appropriate.

Mr. Adam Body in the Northern Territory, Australia operated a shrimp farm that included four 2.5 ha ponds, 500 m long and 50 m wide (Chamberlain, 2000). The ponds were divided into 2 raceways, about 25 m wide by earthen baffles. The ponds were each equipped with 4 long arm paddle wheel aerators. Such ponds seem to have many advantages, which are relatively inexpensive and simple. Yet, more experience in planning and operating closed BFT raceways is required.

Green house

Greenhouses are farmed or inflated structures covered with transparent or translucent material large enough under partial or fully controlled environmental conditions for the shrimp or fish culture. In India use of greenhouse technology started only during 1980's and it was mainly used for research activities for agriculture and horticulture purposes. The National Committee on the use of Plastics in Agriculture (NCPA-1982) has recommended location specific trials of greenhouse technology for adoption in various regions of the country. Greenhouse structure of various types are used. Although there are advantages in each type for a particular application, in general there is no single type greenhouse, which can be constituted as the best. Different types of greenhouses are designed as per the location based on shape, utility, material and construction:

The green house can be of wooden framed or pipe framed structure or truss framed structure. The type of covering materials are

- Glass glazing.
- Fibre glass reinforced plastic (FRP) glazing
 - Plain sheet
 - Corrugated sheet.
- Plastic film
 - UV stabilized LDPE film.
 - Silpaulin type sheet.
 - Net house.

Different greenhouse designs are available, ranging from small ponds (a few hundred m) up to structures covering large ponds. Stability of these structures during strong winds may be a problem, calling for a solid and expensive structure. In all cases, ventilation is needed, so as to enable release of heat and allow better temperature control.

Greenhouse raceways for shrimp

Building upon the intensification of lined, outdoor shrimp ponds, member institutions of the former U.S. Marine Shrimp Farming Consortium developed biofloc technology in intensive lined raceways in standard greenhouses (100 feet long \times 25 feet wide). These greenhouses can be sited inland to avoid expensive coastal land and in areas with a temperate climate if supplemental heat is provided. Experimental or nursery-scale raceways (40 to 50 m³) and commercial-scale systems (250 to 300 m³) are constructed to fit in a standard greenhouse. Raceways are shallow (about 50 to 100 cm) and typically include a central baffle or partition to improve internal circulation. Water movement is provided by banks of air-lift pumps that draw water from the tank bottom and release it at the tank surface or by pumps that inject water through nozzles designed to provide aeration. Water is directed to flow along the tank in one direction and in the opposite direction on the other side of the partition. Raceways also have an extensive network of diffused aeration to maintain biofloc in suspension. At the highest intensities and standing crops, oxygen may be injected for a short time after feeding or continuously as needed.

Biofloc solids concentration is managed with settling tanks. Settling tank volume is less than 5 percent of system volume. Some systems include foam fractionation to capture fine solids and foam. Best operation occurs when settleable solids are 10 to 15 mL/L; best shrimp feed consumption occurs at the low end of that range. Shrimp (SPF) juveniles are stocked at 300 to 500 PL per m² (up to 750 to 1,000 PL per m²). Yields of 3 to 7 kg/m² are typical, with yields of 10 kg/m² possible with pure oxygen supplementation. Water use is about 200 to 400 L/kg. In addition to shrimp grow-out, biofloc technology can be used in commercial nursery systems. The relatively small and shallow raceway is physically suitable for intensive nursery culture. Importantly, juvenile shrimp may be able to take better advantage of the nutritional benefits of biofloc than larger shrimp.

Greenhouse raceway for shrimp (Clemson system)

A variation of a shrimp biofloc system in a greenhouse has been evaluated at Clemson University. The system consists of three shrimp rearing tanks, each of which is 250 m², containing 150 m³ of water. The system is operated with a solids concentration of 200 to 500

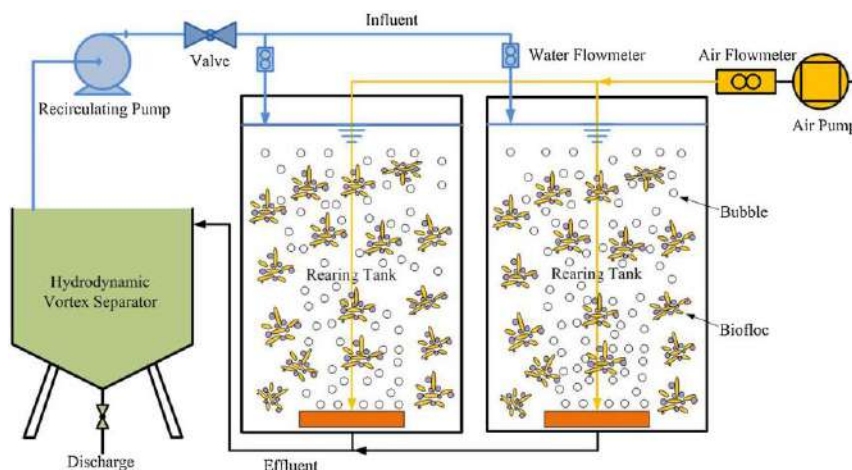
mg/L (15 to 50 mL/L). Water from rearing tanks flows to a primary solids settling tank where it is allowed to become anoxic. Denitrification and some alkalinity recovery occur here under those conditions. Water then passes to an aerated tank stocked with tilapia, which provide filtration (polishing) and nutrient recovery. Next, water flows into an intensively mixed tank with dense biofloc (1,000 to 2,000 mg/L) that serves as a biofilter to oxidize ammonia. Water then flows to a tank for solids settling before returning to the rearing tank. Settled solids are recycled to the suspended-growth biofilter. The main difference between this and the previously described system is the use of a dense suspension of biofloc separate from the shrimp as a biofilter. The Clemson system is also different in that it includes an anaerobic component in the treatment loop. The system has produced 2.5 to 3.5 kg/m² in a 150- to 180-day growing season. Sustainable feeding rates in excess of 1,000 kg/ha and peak



Raceway system

Recirculatory Biofloc system _ RBFT

A different approach, recirculating aquaculture systems, RAS, is based upon the treatment of water quality in a separate compartment using mechanical solids separation, biofilters of different types and often water sterilization,



The rearing tanks are cone-shaped 630 mm high, 420 mm bottom diameter, 500 mm top diameter, and average water depth of 550 mm. All the tanks have a 25 mm diameter drain in the bottom center, while 20 mm diameter inlet is located in each tank's wall and 10 mm below the water surface. In each tank, there is an annular air diffuser (porous rubber with an external diameter, 16 mm and internal diameter, 8 mm, length, 650 mm each) and a 2-Hp aerator (Blower) to provide air and to keep the solids suspended. The circulating pump offered the power to make sure the recirculation of RBFT system, which results in a velocity of the inlet water (the regulation of water inlet velocity is based on valve and water flowmeter). The inlet velocity greatly changed the flow pattern of the rearing tank, which could affect the distribution of biofloc particles, just like the size of bubble supplied by aerator. To a certain water inlet velocity and bubble size, the rearing tank could obtain a homogenous distribution of biofloc. According to the homogenous distribution, the TSS of rearing tank would decrease to an expected level because HDVS could discharge the biofloc particles effectively and exactly.

Conclusion

High-density rearing typically requires some waste treatment infrastructure. At its core, biofloc is a waste treatment system. Biofloc systems were also developed to prevent the introduction of disease to a farm from incoming water. Shrimp farming began moving toward more closed and intensive production where waste treatment is more internalized. Super-intensive shrimp culture systems require a somewhat unique set of engineering design and management criteria. Many of these issues are still being explored by the scientific community as well as by the aquaculture industry. The future surely holds a place for super-intensive biofloc systems or some adaptation of this technology if responsible aquaculture development is to progress.

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BIOFLOC TECHNOLOGY FOR WATER QUALITY MANAGEMENT IN AQUACULTURE

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Introduction

In aquaculture systems, much of the nitrogen input enters the water column as total ammonia-nitrogen generated by feed, which is not converted into shrimp tissue. Generally, metabolites like ammonia and nitrite are generated during intensive aquaculture as a consequence of aquatic animal excretion, sediment mineralization and microbial process in water column. Nitrogen loss varied from 14.7% to 34.7% based on stocking density under zero water exchange culture system. The presence of TAN in water above 1.0 ppm can cause adverse health effects in aquatic animals and create environmental concerns if effluent is not properly treated. The metabolites are deleterious to the animal which causes increase in blood pH, reduction in oxygen content of blood, affects gills, causes stress, reduce feeding, retarded growth, poor survival and high susceptibility to disease. Many biological treatment systems have been developed to maintain ammonia and nitrite concentrations in culture water.

Pathways to control ammonia in aquaculture pond environment

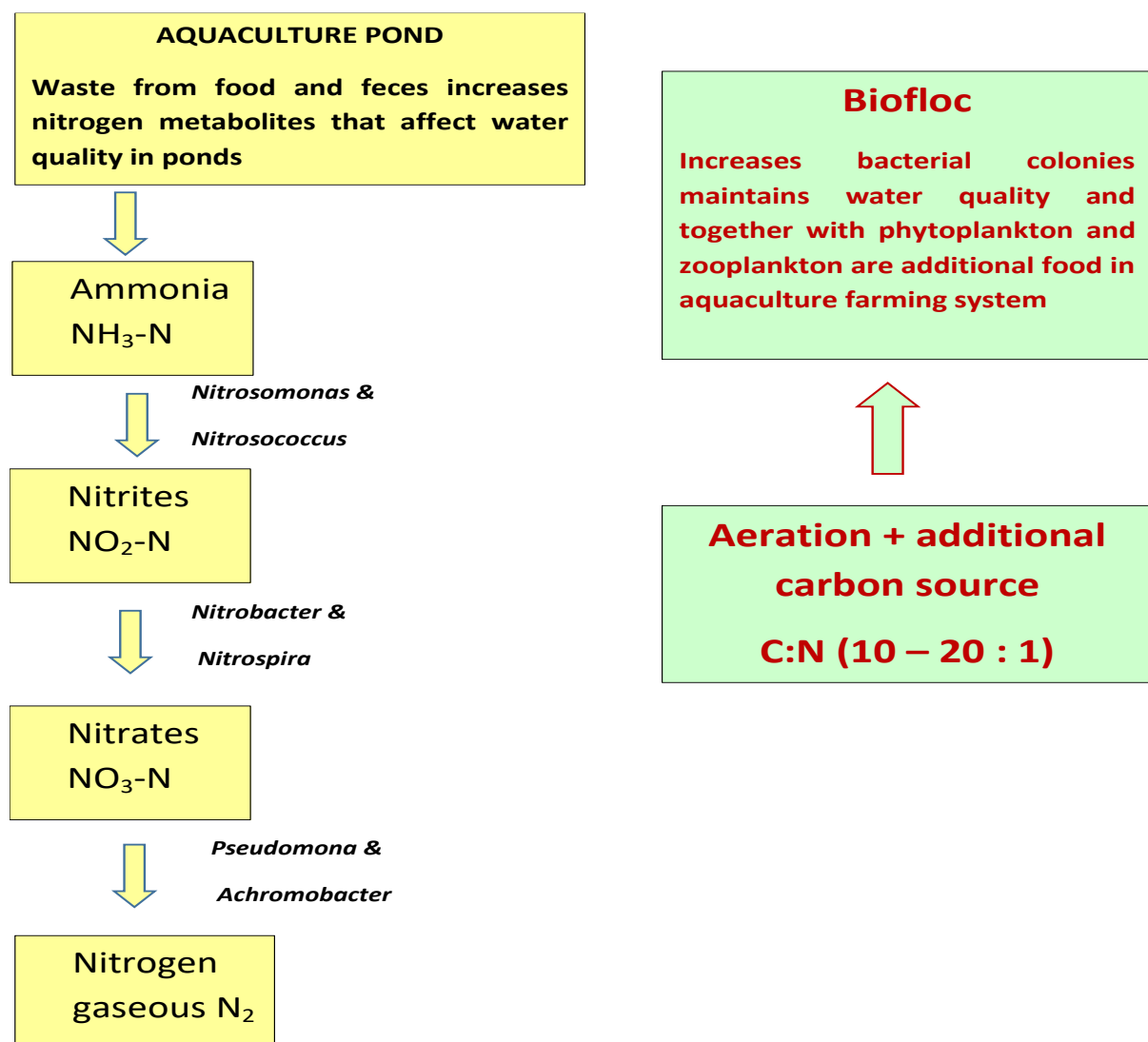
The three nitrogen conversion pathways by microbes for removal of ammonia nitrogen in aquaculture system are photoautotrophic removal by algae, autotrophic bacterial conversion of ammonia nitrogen to nitrate nitrogen and heterotrophic bacterial conversion of ammonia nitrogen directly to bacterial biomass (Biofloc). Phytoplankton based systems are attractive because of their simplicity and low operational cost but fail to sustain a stable operation because of periodic phytoplankton bloom and crash cycles. Nitrifying bio filters have been successfully employed in various aquaculture applications. Despite many advantages, the use of nitrifying bio filters remains costly. Currently, biofloc technology systems have been receiving attention because they feature high production, water quality control, and feed protein recycling simultaneously in the same culture unit. Biofloc system leads to sustainable production through reduction of cost of feed and water exchange and protect the surrounding environment.

Biofloc Technology

The Biofloc technology (BFT) is based on the manipulation of microbial community through the addition of a carbon source that promotes the development of heterotrophic bacteria. These bacteria use the organic carbon and the inorganic nitrogen present in the water to produce their biomass by removing toxic ammonia from the culture system. The basic principle of the BFT system is the retention of waste and its conversion into biofloc as a natural food source within the culture system. This system facilitates the production of aquatic animals at high stocking densities in a sustainable and bio-secure fashion. In some cases, the protein content of feed can be reduced due to partial protein supplementation by the microbial community.

Biofloc as a bioremediator to improve water quality

Biofloc is nothing but aggregates of bacteria dominated by heterotrophic, organic material, inorganic flocculants and suspended algae. All of these microscopic organisms have their own function and interact between each other in the biofloc system to make the bioremediation process successful and maintain water quality during the culture. The algae serve as a food for the animal/pond stock and the bacteria promotes direct conversion of nitrogenous waste to simpler compounds. This process maintains or greatly improves the quality of pond water. Improvement in water quality drastically reduces the need to use large volume of additional water in the pond. This leads to sustainable activity that is in balance with the environment and reduces the cost of water and feed for the pond stock. The biological processes in biofloc system to improve water quality are shown in the figure below.



Factors controlling ammonia concentration in Biofloc Aquaculture System

1. Balancing input C:N ratio

In biofloc systems, a major factor that controls ammonia concentration is the C:N ratio of feed and other inputs. A feed with a 30 to 35 percent protein concentration has a relatively low C: N ratio of about 9 to 10:1. Increasing the C:N ratio of inputs to 12 to 15:1 favors the heterotrophic pathway for ammonia control. The low C: N ratio of feed can be augmented by adding supplemental materials with high C:N ratio or, the input C:N ratio can be increased by reducing feed protein content. At high C:N ratio, heterotrophic bacteria dominate autotrophic microorganisms, immobilize the ammonium ion for production of microbial protein and maintain inorganic nitrogen level in the water within the limit. Immobilization of ammonium by heterotrophic bacteria occurs much more rapidly because the growth rate and microbial biomass yield per unit substrate of heterotrophs are a factor 10 higher than that of nitrifying bacteria.

2. Type of carbon

Capacity of the biofloc system to control water quality in the culture system and the nutritional properties of the flocs are influenced by the type of carbon source used to produce the flocs. Some of the carbon sources are tapioca flour, wheat flour, molasses, sugar etc. Generally molasses addition was an effective tool in controlling these nitrogen compounds.

3. Amount of carbon

The amount of carbon addition depends on several factors such as water quality, physiology and growing pond density of the animal, quality and quantity of animal and solubility of carbon source. To operate biofloc system efficiently, C:N ratio has to be maintained in the ratio between 10:1 and 20:1. Under optimum C:N ratio, inorganic nitrogen is immobilized into bacterial cell while organic substances are metabolized.

4. Aeration and mixing of water

Constant intensive turbulent mixing is essential in a BFT system in order to keep the solids suspended in the water column at all times. Without mixing, bioflocs can settle out of suspension and form dense piles that rapidly consume nearby dissolved oxygen, creating an anaerobic zone. These zones can lead to the release of chemical compounds such as hydrogen sulphide, methane and ammonia that are toxic to shrimps and fish. In practice, aeration is used to supply oxygen and provide adequate mixing. Various configurations of aeration equipment are possible, depending on the specific form of biofloc system. In lined ponds or tanks, multiple paddlewheel aerators are arrayed to provide whole-pond, circular mixing. Shrimp raceways in greenhouses often use banks of airlift pumps placed at intervals around raceways to aerate and circulate water. Diffused aeration can be used in small tanks. Devices that circulate water at low head, such as low-speed paddlewheels and airlift pumps, can be used. Biofloc shrimp ponds are aerated with 25 to 35 hp/ha, and some intensive tilapia systems are aerated with 100

to 150 hp/ha. These intensive aeration rates could not be applied to earthen ponds without significant erosion; thus, most biofloc systems are lined. Biofloc systems are not a good choice in areas where power supplies are unreliable or electricity is expensive.

Changes in water quality under biofloc system

Under biofloc system, ammonia and nitrite concentration are generally low due to the removal of these compounds by microbial community. Nitrate concentration was low due to the lower concentration of ammonia nitrogen available to the oxidation by nitrifying bacteria. The absorption of the reduced form of inorganic nitrogen by phytoplankton is probably the primary cause for high concentration of Chlorophyll a concentrations in this system. The use of carbon sources in intensive systems promotes succession and dominance of bacteria over microalgae.

Physical-chemical characteristics of water under biofloc system

Temperature

Temperature is one of the most influential parameters in pond system. It affects metabolic rate of animal & microorganism, oxygen consumption, pH and concentration of ionized and un-ionized ammonia during culture. The optimum temperature range will depend on the animal species, bacteria adapted to the system temperature as well as seasonal and environmental variations. Biofloc system is more efficient when water temperature is between 28 and 30°C. Nitrifying bacteria can support a range from 8-30°C, but efficiency is reduced by 50% at 16°C and by 80% at 10°C.

Dissolved oxygen

Oxygen in aquatic system should be > 5 ppm. In a biofloc system, as the algae and bacteria also have oxygen demand, dissolve oxygen should be maintained at 7-8 ppm to ensure proper functioning of the system.

pH

pH is to be maintained in the range between 7 and 8.5. Hydrated lime ($\text{Ca}(\text{OH})_2$) is to be used to maintain alkalinity and pH above 100 ppm of CaCO_3 and 7.5, respectively in the biofloc system. pH reduction generally occurs due to alkalinity consumption during ammonia–nitrate nitrogen conversion processes. According to Furtado *et al.* (2011), levels under 100 ppm of CaCO_3 and pH 7 for prolonged periods of time can affect the growth performance of shrimp in biofloc.

Alkalinity

Alkalinity is the capacity of water to buffer or resist changes in pH in response to additions of acid or base. Water in biofloc systems should be maintained with ample reserves of alkalinity because it is constantly depleted by the activity of nitrifying bacteria in nitrification. Once alkalinity is depleted, pH can drop steeply, inhibiting bacterial function,

including important nitrifying bacteria. In this situation, ammonia accumulates to the point where fish appetite and feeding response will deteriorate. This limits daily feeding rate, feed conversion efficiency and ultimately production. Alkalinity should be kept between 100 and 150 ppm as CaCO_3 by regular additions of sodium bicarbonate. Other liming agents are less suitable. Every kilogram of feed added to the system should be supplemented with 0.25 kilogram of sodium bicarbonate.

Suspended solids, settleable solids and volatiles

Bacteria depend on suspended solids as a substrate for adhesion and as a source of energy from carbon. In biofloc system, TSS in the range of 250-450 ppm ensures efficient bacterial activity and a good system to control ammonia without excessive water respiration. An excess of TSS affects the breathing process of animals, lead to stress or in extreme cases, lead to death by clogging gills. Cultivation of *L.vannamei* in biofloc system contained 453 ± 50 ppm of TSS and 256 ± 106 ppm of volatile solids improved shrimp production provided efficient exchange of oxygen. The desired range of settleable solids concentration is 10 to 15 mL/L for shrimp and 25 to 50 mL/L for tilapia under good biofloc system.

Turbidity

In aquaculture system, turbidity is due to suspended solids, phytoplankton, zooplankton and bacteria. Turbidity is measured by Secchi disk and the value of 35-40cm is acceptable. Turbidity of 75 to 150 NTU is comparable to the recommended settleable solids concentration provided that color interference is not too severe.

Total Ammonia Nitrogen

It is the excretion product of feces, urine, uneaten food, phytoplankton and zooplankton. Ammonia or non-ionized ammonia (gaseous) is considered as toxic when compared to ionized ammonia or ammonium ion (NH_4). The unionized form (NH_3) increases with a low oxygen concentration, high pH and high temperature. The recommended ammonia concentration in biofloc culture is less than 1.5 ppm. In *L.vannamei*, ammonia nitrogen concentration should be less than 1.2 and 6.5 ppm in post-larvae and juveniles.

Nitrite nitrogen

The transformation process to ammonia nitrogen to nitrite nitrogen and their toxicity form depends on the amount of chlorides, temperature and oxygen concentration in water. Nitrite toxicity affects transport of oxygen, oxidation of important compounds and tissue damage. Nitrite-nitrogen concentration should be less than 2 ppm in biofloc culture.

Nitrate nitrogen

It is the end product of aerobic nitrification and considered as less toxic. The toxicity of these compounds is due to its effects on osmoregulation and oxygen transport. Nitrate concentration should not exceed 10 ppm in biofloc culture.

Promising features of biofloc technology

Water is becoming scarce or expensive to an extent of limiting aquaculture development. Secondly, the release of polluted effluents into the environment is prohibited in most countries. Thirdly, severe outbreaks of infectious diseases led to more stringent biosecurity measures, such as reducing water exchange rates (Crab *et al.* 2012). Biofloc technology addresses the above issues making it a promising environment friendly solution.

Benefits accruing in pond water quality due to the use of biofloc technology

1. Improving water quality through removal of toxic nitrogen compounds such as ammonia and nitrite (Schryver *et al.* 2008; Ray *et al.* 2011).
2. The water quality of a heterotrophic microbial-based production system containing bacterial flocs is more stable than that of a phytoplankton-based production system Boyd and Clay (2002).
3. Biofloc technology makes it possible to minimize water exchange and water usage in aquaculture systems through maintaining adequate water quality within the culture system (Crab *et al.*, 2012).
4. Compared to conventional water treatment technologies used in aquaculture, biofloc technology provides a more economical alternative by reducing the water treatment expenses to the tune of about 30%.
5. Enhancing the farm biosecurity and health management through zero-water exchange and possible probiotic effect (Crab *et al.* 2010; Zhao *et al.* 2012).

Conclusion

Aquaculture is expected to grow at a rapid pace all over the world in the coming years, hence there is a need to improve production, productivity while reducing the cost of culture and protecting the environment. Biofloc offers a lucrative combination of protecting the pond environment using natural resources, reducing the input cost of feed and effectively managing the additional water requirement, thereby providing an all-round economically feasible solution. In the future, biofloc will be one of the major technologies that will be widely utilized for pond water quality management.

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BMP FOR POND SOIL MANAGEMENT

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A pond with good soil quality will produce more of healthier shrimp than a pond with poor quality. Understanding of the soil properties helps in selecting a site for culture, and to decide the management strategies to be followed in terms of liming, manuring, fertilization, water management etc. A satisfactory pond soil is the one in which mineralization of organic matter takes place rapidly and nutrients are absorbed, held and released slowly over a long period. Sandy clay, sandy clay loam and clay loam are some of the textured names suitable for aquaculture. Soil organic matter is an important index of soil fertility and also helps in prevention of seepage loss, increases arability of pond soil bottom and supplies nutrients. The soil rich in CaCO_3 content promotes biological productivity as it enhances the breakdown of organic substances by bacteria creating more favourable oxygen and carbon reserves. Most of the problems such as low organic matter content or excessive porosity can be avoided by proper management practices during pond preparation.

Pond preparation

The main objectives of pond preparation are to provide the shrimp with a clean pond base and appropriate stable water quality. In newly dug out ponds, Soil samples taken from different locations of the pond are thoroughly mixed together and a representative portion has to be taken for analysis. Soil deficiencies should be identified and treated in new ponds instead of waiting until poor bottom soil quality develops later. In the ponds after harvest, drying, ploughing, and liming are required to correct the pond soil conditions with regard to the accumulated nutrients and acidity. In high density cultures, there is a possibility of the pond sediment becoming unusable during the next culture due to high accumulation of nutrient loads. Disposal of such sediments outside will be an environmental hazard.

In ponds with high sand content or desired to have high productions high stocking density like biofloc culture, polythene lining is preferred. Monitoring of *P.vannnamei* (60 /m²) culture in low saline (2 ppt) plastic lined ponds indicated optimum range of metabolites concentration (TAN - 0.02 to 1.05 ppm; Nitrite-N - 0.003 to 0.17 ppm) during culture period. Though only 60% survival was obtained due to low salinity and minerals concentration (Calcium – 30 to 79 ppm and Magnesium – 51 to 72 ppm), production of 7 t/ha was achieved with FCR of 0.8.

Drying, liming and fertilisation

Removal of waste from the previous crop by draining and drying of the pond bottom after the production cycle are some of the steps to be followed for keeping pond environment

clean. The sludge left in the pond, which might have had viral disease outbreak during the previous culture, may contain high organic load, bacteria, viral particles and DNA as well as many other viral carriers. All these can be removed by the application of burnt lime (CaO) @ 100 ppm, followed by exposure of the pond bottom to sunlight for a minimum period of 21 days until it dries and cracks, removal of the top soil and compacting the bottom soil to prevent the persistence of viral disease. Soil respiration measured in a pond bottom increased drastically during first 3 days after drying. The optimum moisture content for drying is 20%, but it might vary among soils from different ponds.

The reason for liming aquaculture ponds is to neutralize soil acidity and increase total alkalinity and total hardness concentrations in water. This can enhance conditions for productivity of food organisms and increase aquatic animal production. Either total alkalinity or soil pH may be used to estimate the agricultural limestone dose. If both the values are not in agreement, use the variable that gives the greatest agricultural limestone dose. Brackishwater ponds with total alkalinity below 60 mg l⁻¹, and any pond with soil pH below 7 usually will benefit from liming.

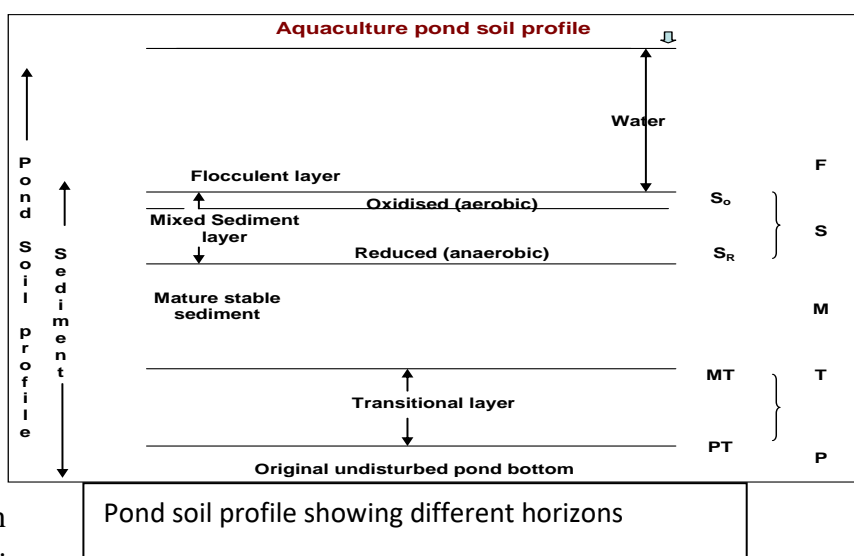
Application of organic fertilizers especially in newly developed ponds is advisable because it serves as soil conditioner. The rate of application of organic manure in shrimp ponds ranges from 500 to 2000 kg/ha as a basal dose. Enhancement of nutrients using inorganic fertilisers is required in ponds to increase the phytoplankton production. Decomposition of organic matter in harvest pond soil is slow because pH usually is low and the amount of carbon relative to nitrogen (C:N ratio) is high. Urea can be spread over pond bottom @ 200 to 400 kg ha⁻¹ at the beginning of the fallow period to accelerate decomposition. Sodium nitrate can be applied @ 20 to 40 g m⁻² to wet soil to encourage organic matter decomposition in wet areas. However, nitrate fertilizers are more expensive and are not recommended where soils can be adequately dried.

Management of pond bottom during culture

All aquaculture pond bottoms become covered with sediment, and this sediment can be considered as aquaculture pond soil. A core taken through the sediment and extending into the original bottom soil is called a profile. Layers in the profile are known as horizons (Fig.1). For practical purposes, the F and S horizons are most important in aquaculture because they exchange substances with overlaying water to influence water quality.

Oxidized Layer

The oxidized layer at the sediment surface is highly beneficial and should be maintained throughout the shrimp culture. Anaerobic microorganisms are able to use oxygen from nitrate, nitrite, iron and manganese oxides, sulfate, and carbon dioxide to decompose organic matter, and release nitrogen gas, ammonia, ferrous iron, manganous manganese, hydrogen sulphide, and methane as metabolites.



Some of these metabolites, and especially hydrogen sulfide and nitrite, can enter the water and be potentially toxic to shrimp. It is extremely important to maintain the oxidized layer at the sediment surface in shrimp culture ponds. Ponds should be managed to prevent large accumulations of fresh organic matter at the soil surface, or in the upper few millimeters of soil. If the rate of release of toxic metabolites into water exceeds the rate that metabolites that are oxidized, equilibrium levels of metabolites in the water may be high enough to have detrimental effects on culture animals.

Monitoring of soil parameters during culture period

During culture the carbonaceous matter, suspended solids, faecal matter and dead plankton etc. settle at the pond bottom. Interactions between soil and water that influence water quality in ponds must not be ignored, because poor soil condition in ponds can impair survival and growth of aquaculture species. Major concerns in pond bottom soil management are low soil pH, high soil organic matter, loss of the oxidized layer, and accumulation of soft sediment. In older ponds with impaired soil quality, problems should be corrected and prevented from recurring. To understand the condition of the pond bottom, the following parameters are to be monitored regularly.

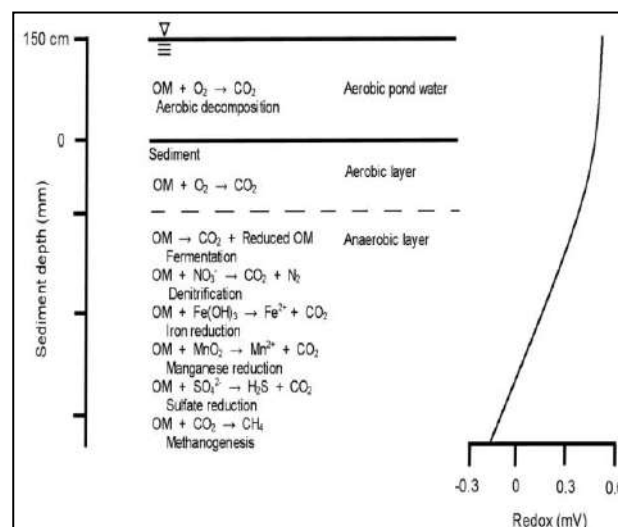
Organic matter

The change in the bottom in terms of increasing organic load should be recorded regularly for the management of the pond bottom.

Redox-potential

Anaerobic condition can be developed in pond, when input of organic matter exceeds the supply of oxygen needed for decomposition of organic matter. This reducing condition can be measured as the redox potential (E_h). Redox potential indicates whether the water or soil is

in reduced (E_h with '-' value) or oxidized (E_h with '+' value) condition. Reduced or anaerobic sediments may occur at the pond bottom of heavily stocked pond with heavy organic load and poor water circulation. Chain dragging once in fortnight improves the pond bottom condition by maintaining the oxidised layer. However, it has to be started from the initial period of culture. Central drainage system, an ideal remedy for the prevention of formation of highly reducing condition during the last phase of culture period is suggested to remove organic waste periodically. Water circulation by water exchange, wind or aeration helps to move water across mud surface and prevent the development of reduced condition. Bottom should be smoothened and sloped to facilitate draining of organic waste and toxic substances. The redox potential (E_h) of mud should not exceed -200 mV.



Aerobic and anaerobic reactions at the pond bottom soil



On-farm measurement of redox potential

AERATION REQUIREMENTS IN BIO FLOC BASED SHRIMP CULTURE SYSTEMS

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Adequate dissolved oxygen is a very important parameter for successful intensive aquaculture as it affects the quality and quantity of growth. Artificial aeration is provided to increase the oxygen concentration and also to remove the anoxic gases such as nitrogen and carbon dioxide. The higher crop biomass and feeding rates of intensive shrimp culture systems require supplemental aeration to maintain adequate dissolved oxygen levels (above 4 ppm). Farmers adopting high-density culture are in constant fear of low DO instances and tend to use more aeration as there is no available information on optimum aeration requirement at different stages of culture and different stocking density. Too little aeration leads to a hypoxic condition in ponds resulting in mass mortalities and more aeration results in excessive operating expenses and wastage of energy. Shrimp die when DO concentrations fall below 1mg/L for more than few hours and also if DO level goes below 4 mg/L for several hours, it will create severe stress on animals. Less than the optimum levels of DO can lead to poor water quality, feed conversion ratios (FCR), reduced growth or survival.

Aeration requirements for different density systems

Extensive /traditional systems do not need any artificial aeration as the DO in the pond or waterbody is sufficient to meet the animal requirement. When the stocking density goes high, the bottom layer of the pond water becomes hypoxic or anoxic especially at night hours due to higher respiration rate and decomposition of accumulated organic matter

Aeration is the mechanism to maintain the aquatic animal free from oxygen deficiency stress and helps to maintain water circulation. It is the mass transfer phenomena between air and water, of which the variation of the oxygen concentration in the water as a function of time. Hypoxia or low DO (less than 2.8 mg/L) can certainly threaten shrimp life. DO values higher than 5 mg/L have been recommended in intensive culture practices. It has been reported that the lethal DO levels ranged from 0.2 to 1.27 mg/L for several penaeid shrimps.

In high-density biofloc based farming, intensive turbulent mixing is very much essential for biofloc systems as solids must be suspended in the water column at all times to make the system function. Important requirement in the bio-floc system is to meet high oxygen

demand. Typical aeration requirements are 1 HP/300-350 kg shrimp biomass. The bio-floc system requires high aeration/mixing with aerators, and blowers not only to provide DO and remove CO₂ (70-90ppm) but also to keep bio-floc in suspension and prevent the production of nitrogenous metabolites, sulfides and organic acids. Also, direct an aspirator type aerator at the sludge pile to re-suspend it. Optimum uninterrupted aeration is essential to maintain high DO whereas less turbulence will lead to the production of hydrogen sulfide, methane, and ammonia and more turbulence will make it difficult for shrimp to locate feed or can lead to pond dike aeration. Water respiration in indoor biofloc systems is normally about 6 mg O₂/L per hour. It is believed that aeration requirement of 1 HP of every 200-250 kg of biomass.

Aeration methods

There are three categories of aeration methods, viz., surface or mechanical aeration, diffused aeration, and combined or turbine aeration method. Mechanical aerators such as paddle wheels, vertical pumps, pump sprayers, propeller aspirator pumps, and diffuser air systems are widely used because of better efficiency and convenience of operation. Stationary paddlewheel aerators are commonly placed parallel to the dikes of the pond, creating a radial or elliptical water flow regime with rapid water flow in the periphery and stagnant conditions in the center.

Estimation of aeration requirements

Estimating supplemental aeration needs to be done considering the biomass and the feeding rate. Early attempts in *L.vannamei* ponds were based on Taiwanese rule of thumb” for intensive *P.monodon* culture. This indicates that one horsepower of aeration per 650 kg of anticipated harvest biomass. There is a real danger in basing the aeration rates on anticipated harvest mass. If higher than the normal feed rates for given projected biomass is used, critically low oxygen levels can be experienced.

Aeration requirements depend on the stocking density, feeding rate, and early morning dissolved oxygen in shrimp ponds, and it is related as:

$$F/A = 28.83 - (4.31 \times DO)$$

where F = 5-day average feeding rate (kg/ha/day); A = aeration rate (hp x day/ha); DO = dawn DO concentration (mg/L). These relationships will facilitate economic optimization based on capital equipment expenses for electricity, seed, and feed. Supplemental aeration may be needed in shrimp ponds at feed rates above 40-50 kg/ha. day. *L.vannamei* shrimp is

cultured under intensive culture, and it is column dwelling species, so the oxygen has to be distributed throughout the pond.

Aerators use in shrimp farms

Aeration with better oxygen transfer rate is very much essential to reduce the production cost and maintain the good shrimp culture. The placement of aerators in the pond assumes greater importance given the need to maintain proper water circulation for maintaining feed and biofloc in suspension and also concentrate the waste in one place. While feed accounts for nearly 50-60% of the total recurring cost, the electricity and diesel charge nearly take away 25 to 30% of the recurring cost. The electrical power supply has become very erratic and costly. Diesel engines are commonly used to run aerators that further add input cost.

Different types of paddle wheel aerators with custom made the design are widely used in shrimp ponds with different length, capacity, No. of blades, source of power, and placement. In some places, small farmers have changed the paddle wheel aerators to custom made one and operated using the diesel engine due to non-availability of electricity. The efficiency and energy consumed per kg of production using different types of aerators are either not known or not evaluated to optimize the best design to produce maximum oxygen transfer rate with less energy. Few other types of aerators such as injector types, diffuser types are also being tried by a few farmers. Monitoring the efficiency of the aerators in farmer's ponds will pave the way for optimum design criteria that can deliver maximum oxygen transfer rate with less energy in another way with a reduction in operation cost.

Oxygen demand and aeration requirement increase with culture period, but there is no system adopted by the farmers to gradually increase the aeration hours. Operation costs demand the correct calculations to optimize the type of management used and the region of culture. Estimation of aeration requirement for each week of culture can bring substantial savings with electricity and consequently production costs. The study from CIBA indicated that energy used per kg of shrimp production varied widely and too little or much aeration is given in many ponds as no information on exact aeration requirement is available for different stocking densities of different species and pond conditions. In the shrimp farms, the hours of operation of aerators vary from farms, and there is no standard procedure or mechanism adopted based on the biomass.

Different aerators in shrimp ponds

Paddlewheel aerators: Semi-intensive / intensive ponds are commonly aerated by placing the paddle wheel aerators parallel to the pond dikes, creating a radial or elliptical

water flow regime with rapid water flow in the periphery and stagnant conditions in the center. Water is pumped into the air by pushing into the air with the standard water transfer efficiency of 1.7 kg and consumes the energy of 1HP /hr for 2PW aeration. Normally 1hp aeration is given for 300 to 400 kg of biomass. The aeration requirement is more when the level of water exchange is reduced, which has become common as a means to reduce the probability of pathogen transfer. Normally 2, 4 or 6 wheel aerators are used. Paddlewheel, long arm and spiral were three kinds of aerators found adopted by the farmers. In some places, small farmers have changed the paddle wheel aerators to custom made one and operated using the diesel engine due to non-availability of electricity.



Custom made 7 wheel and 6 wheel PWA with diesel engine and modified car engine



Aero tubes



Round flow pond aerator

Fig. 1Types of aerators

Different types of two, four, seven, eight wheels operated with 3 phase electric motor or customized car engines (Diesel) were used in the farms. Motors for paddle wheel aerators usually turn at 1750 rpm, but this speed is reduced so that the paddle wheel rotates at 70–120 rpm. There is considerable variation in the design of the paddle wheel and in the

mechanism for reducing the speed of the motor output shaft. Many studies indicated the variation in aerator efficiency at varying salinities.

Water circulation may cause soil erosion in intensive shrimp ponds unless banks are protected. Aeration of marine shrimp ponds should be arranged in such a way as to sweep the feed area of the pond. Each 1 kW of aeration will support an additional 500 kg shrimp production, beyond a base of 2000 kg/ha that may survive by natural re-aeration. Square ponds are easier to manage because four paddlewheels can be arranged to wash out the corners.

Round flow pond aerator

This type of aeration devices can be used to increase oxygen levels during feeding. The oxygen transfer efficiency was measured as 3 kg oxygen transfer the available capacity ranged from 0.5 to 5 HP.

Injector type submersible aerator



Fig.2 Injector type aerator

This injectors are one of the best and most advanced aeration systems worldwide, and also suitable for large ponds. They work with a for an endurance run produced, maintenance free, adjustable, submerged (also seawater resistant), the motor in different power sizes (1.1-1.5 kW (1.5-2.0 HP) 230 or 380 V/50 Hz). The rotating propeller (2800 r.p.m.) produces an adjustable current, through which air is drawn from the surface and formed into fine evenly distributed bubbles. The diffused air aerate from the bottom up, and the circulation created from the fine bubbles displace the surrounding water, thus breaking up stratified water areas. This constant surge forces the pond to turn over, bringing cooler bottom water to the surface, where it picks up additional oxygen from the atmosphere.

Therefore the dissolved oxygen is always as high as possible. Depending on power and depth, the air flow rate is up to 35 m³/hour and the oxygen introduction up to 3

kg/kw/h/. At an oxygen content of 6 mg O₂/L at the beginning, one 1.0 HP unit in use with air, introduce over 0.32 kg O₂/h which is the oxygen demand for over 2600 kg (table sized 250 g) trouts at a water temperature of 10 °C, for approx. 1000 kg at 20 °C or up to 3000 kg of other species

Spiral aerator

The stackable type is also developed by assembling HDPE floats discretionally, and the metal frame is avoided. It can be assembled according to local voltage and frequency. In aero tubes type, the air is pushed into the water, and less power is consumed while operation. The standard oxygen transfer efficiency is 3.7 kg. It is recommended to use with paddlewheel aerators.



Fig. 3 Spike aerator in shrimp farms

The initial investment cost is more compared to PW aerators. This method gives supplemental aeration in addition to PW aeration and currently being adopted in some shrimp farms and getting popular. However, further validation of the data from different farms is needed to be done to arrive at the exact energy saving from this method.

Blower and air tubes for biofloc



Blower and air tubes are used for aerating chambers in the biofloc system and also the ponds. Compared to the other systems it is proved to be more economical. For e.g. 3HP blower can support 6 grids, for biofloc nursery. Bio floc shrimp culture systems are gaining

importance for cost-effective production where water is limited, or land is expensive. Aeration requirement in bio floc based systems was analyzed based on the primary data collected from the biofloc based shrimp culture ponds at Tamil Nadu and Andhra Pradesh. Air diffuser systems operated using air blowers have been widely used in bio floc systems, whereas different types of surface aerators are used in earthen ponds. Findings revealed that the aeration requirements in the biofloc system vary from 6.2-12 HP/m³ volume of water. Considering the high density per m³ of water, blower system is very economical per kg of shrimp production.

Economic analysis

Fixed cost includes the initial investment cost, depreciation cost, maintenance cost, and bank interest mainly for pond preparation and infrastructure development. Life of aerators can be taken like five years. Variable cost comprises seed, feed, energy, labor, and other consumables. Net profit or loss was arrived based on the difference between the total return and total cost per crop.
$$\text{ROI} = (\text{Net Profit} / \text{Cost of Investment}) \times 100.$$

ROI, NPV, and IRR were calculated from the functions available in Microsoft Excel. NPV and IRR values of shrimp culture using different aerators can indicate the variation in profit at different salinities. NPV should be calculated based on the difference between the present value of cash inflows and cash outflows over a period and indicated the profitability of aquaculture using different aerators. IRR will help us to calculate the variation in profitability among different aerators.

In summary, high-density culture systems like biofloc require efficient aeration mechanisms for which standard aeration efficiency of the aerators at different environmental conditions should be made available. Correct calculation of aeration requirement and energy use can give substantial savings in the production cost.

NUTRITION AND FEEDING STRATEGY IN A BIOFLOC SYSTEM

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Biofloc Technology (BFT) is an environmental friendly aquaculture system since in this farming method nutrients could be continuously recycled and reused. This system is dominated by heterotrophic (HS) microorganisms compared to traditional autotrophic (AS) microalgae. BFT is beneficial by the use of minimum or zero water exchange. The HS has two major roles: (i) maintenance of water quality, by the uptake of nitrogen compounds generating “*in situ*” microbial protein; and (ii) nutrition, increasing culture feasibility by reducing feed conversion ratio and a decrease of feed costs. The water quality of biofloc system is maintained by control of bacterial community over autotrophic microorganisms which is achieved using a high carbon to nitrogen ratio (C:N). Nitrogenous by-products can be easily taken up by heterotrophic bacteria in the presence of adequate quantities of carbon. High carbon to nitrogen ratio (~15-20:1) is thus essential for optimum heterotrophic bacterial growth, using this energy for maintenance (respiration, feeding, movement, digestion, etc) and also for growth and for multiplication to produce new bacterial cells. High carbon quantities in water could supersede the carbon assimilatory capacity of algae, thereby contributing to bacteria growth. Aerobic microorganisms are more efficient in converting feed to new cellular mass (40-60% of conversion efficiency), rather than higher organisms which spend about 10-15% for increase in weight. Bacteria and other microorganisms act as efficient “biochemical systems” to degrade and metabolize organic residues. In other words, they recycle very efficiently nutrients in a form of organic and inorganic matter (un-consumed and non-digested feed, metabolic residues and carbon sources applied as fertilizers) into new microbial cells.

The particulate quantities of organic matter and other organisms in microbial food web are potential food sources for aquatic animals like shrimp. The macro aggregates (biofloc) is a rich protein-lipid natural source available “*in situ*” throughout the day. The BFT is formed in the water column due to the complex interaction between organic nutrients, physical substrate and variety of microorganisms such as plankton, free and attached bacteria, conglomerates of particulate organic matter and also grazers, such as rotifers, flagellates and ciliates and protozoa and copepods. This natural productivity plays an important role for recycling nutrients and maintaining the water quality and providing nutrition to the candidate aquatic species like shrimp.

Biofloc as Nutrient

The consumption of biofloc by shrimp has demonstrated several benefits such as enhancement of growth rate, reduction of FCR and thereby decreased feed costs. Growth

improvement has been attributed to nutritional components of both bacterial and algae. This biofloc nutrition will reduce up to 30% of conventional feeding ration consumption in shrimp. The short-term feeding study using ^{15}N -nitrogen enrichment has demonstrated that bioflocs could contribute substantially to the nutrition of *L. vannamei*. Moreover, the consumption of bioflocs can increase feed utilization efficiency by recovery of some fraction of excreted nutrients and nitrogen retention from added feed by 7–13%. Biofloc technology also helps in rationalizing the feeding rates. The results of feeding rates of (10%, 9.5%, 9.0%, 8.5%, and 8.0% body weight day⁻¹ in biofloc system) were compared with control at 10% feeding level but without biofloc indicated that 8% feeding rate performed on par with control.

In tilapia feed utilization is higher in BFT at a rate of 20% than conventional water-exchange systems. These results of BFT have driven opportunities to use alternative diets. The feeds were formulated with low nutritional densities especially for intensive biofloc systems. This has increased the scope to reduce protein levels in the feeds and also to use alternative feed ingredients by replacing different marine protein sources (i.e. fishmeal, squid meal, etc) leading “green” market opportunities.

In biofloc particles, protein, lipid and ash contents vary substantially (12 to 49, 0.5 to 12.5 and 13 to 46%, respectively. The same trend of wide variation also occurs with fatty acids (FA) profile. Essential FA such as linoleic acid (C18:2 n-6 or LA), linolenic acid (C18:3 n-3 or ALA), arachidonic acid (C20:4 n-6 or ARA), eicosapentanoic acid (C20:5 n-3 or EPA) and docosahexaenoic acid (C22:6 n-3 or DHA), as well as sum of n-3 and sum of n-6 differ considerably between 1.5 to 28.2, 0.04 to 3.3, 0.06 to 3.55, 0.05 to 0.5, 0.05 to 0.77, 0.4 to 4.4 and 2.0 to 27.0% of total FA. Type of carbon source, freshwater or marine water and production of biofloc biomass (in bioreactors or culture tanks) definitely influence the FA profile. Information is still scarce about how microorganisms profile and its nutritional contents could impact shrimp growth.

The amino acid composition of the biofloc produced from different carbohydrate sources like maida, wheat, gram flour, ragi, rice flour, corn flour, molasses and multigrain atta. The result revealed higher level of essential amino acids in ragi, molasses and multigrain atta compared to other treatments. The utilization of protein by the shrimp depends on the availability of essential amino acids in the diet. Among them certain amino acids like arginine, methionine and lysine are limiting amino acids as they are generally deficient in the diet. The biofloc samples from ragi, molasses and multigrain atta are having higher arginine, methionine and lysine levels of 1.35, 1.12, 2.13; 1.32, 1.17, 1.66 and 1.25, 1.093, 1.663 per 100 g dry floc, respectively compared to other treatments and control. Because of higher essential amino acids, the essential amino acid index is also better in biofloc reared with these carbohydrate sources viz., ragi, molasses and multigrain atta.

Table: Amino acid composition (g/100g dry floe) of the biofloc produced with different carbon sources

	C1- Maida	C2- Wheat	C3- Gram Flour	C4- Ragi	C5- Rice flour	C6- Corn flour	C7- Molasses	C8- Multigrain Atta
Essential Amino acids								
Arg	1.143	1.187	1.113	1.353	1.160	1.273	1.320	1.253
His	2.803	2.663	2.740	2.920	2.567	2.440	3.030	2.907
Ile	1.313	1.307	1.193	1.317	1.240	1.173	1.360	1.293
Leu	2.150	2.057	2.050	2.137	1.493	1.727	2.153	1.900
Lys	1.393	1.343	1.463	1.560	1.270	1.257	1.660	1.663
Met	1.007	0.967	1.077	1.127	0.903	0.863	1.170	1.093
Phe	1.547	1.860	1.717	1.803	1.647	1.507	1.773	1.790
Thr	1.653	1.750	1.730	1.870	1.850	1.750	1.810	1.850
Trp	0.290	0.290	0.290	0.330	0.280	0.323	0.367	0.350
Val	1.383	1.373	1.310	1.490	1.373	1.277	1.367	1.313
Non essential amino acids								
Ala	2.677	2.560	2.227	2.267	2.450	2.430	2.287	2.277
Asp	2.863	2.493	2.433	2.553	2.673	2.327	2.367	2.373
Cys	0.417	0.393	0.437	0.453	0.370	0.517	0.457	0.477
Glu	3.277	3.617	3.663	3.407	3.060	3.367	3.433	3.600
Gly	2.183	2.443	2.303	2.150	2.820	3.383	3.463	3.427
Pro	1.290	1.293	1.270	1.287	1.367	1.437	1.290	1.313
Ser	1.350	1.550	1.253	1.383	1.483	1.383	1.440	1.360
Tyr	1.223	1.053	1.133	0.963	1.000	0.987	1.147	1.163

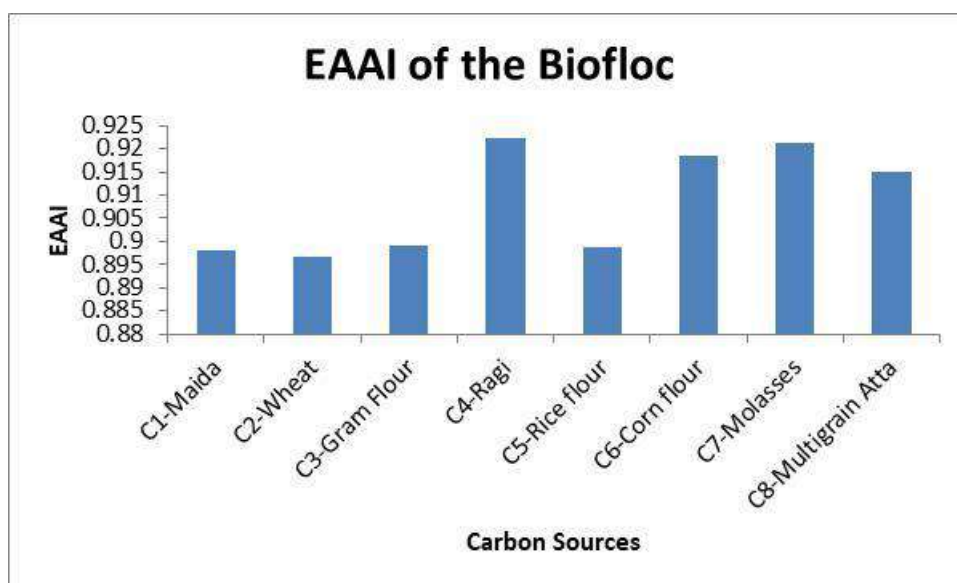


Fig. 1. Essential Amino Acid Index (EAAI) of the biofloc produced from different carbon sources

Role of Biofloc on dietary protein levels in shrimp feeds

Dietary protein levels in the diets of whiteleg shrimp reared in clear seawater system has been studied extensively. Typically commercial shrimp feed contain 38–42% of CP in Asian countries. On the other hand, as far as we know, only few studies have investigated the optimum dietary protein level for whiteleg shrimp reared in bioflocs system. These studies demonstrated the positive contribution of bioflocs aggregates on the optimum dietary protein level in whiteleg shrimp. It was suggested to substitute high protein (40%) with low-protein (30%) feed in the nursery phase. The other study reported dietary protein level could be reduced to 25% without affecting the growth of juvenile whiteleg shrimp reared in bioflocs system. The level of total suspended solids (TSS) also influences the protein level in the diet. Recommended level of TSS was below 500 mg L⁻¹ for shrimp culture.

The bioflocs tanks fed with high dietary CP (40%) than that of low CP (30%) had higher in nitrite, nitrate and phosphate content and TAN content showed increasing pattern with the corresponding increase in dietary protein level, since high-protein feed would have generated more TAN than the low-protein feed ammonia oxidizing bacteria developed faster than nitrite oxidizing bacteria. In the BFT, the formation of the bioflocs was likely to be linked with the direct assimilation of dissolved nitrogenous matters (especially ammonia–nitrogen) from diets and shrimp excretions by heterotrophic bacteria.

It was showed that juveniles of *L. vannamei* fed with 35% CP feed grew significantly better in biofloc conditions as compared to clear-water conditions. The study performed in a heterotrophic-based condition detected no significant difference in FCR when feeding *L. vannamei* 30% and 45% CP diets. These results indicate, floc biomass might provide a complete source of cellular nutrition as well as various bioactive compounds even at high

density. Growth might be enhanced by continuous consumption of “native protein”, protein source without previous treatment, which could possess a “growth factor” similar to the one investigated in squid. Is well known that protein, peptides and amino acids participate fully in synthesis of new membranes, somatic growth and immune function and biofloc can potentially provide all such nutrients.

However, is already known that microorganisms in biofloc might partially replace protein content in shrimp diets, although were not always the case. Recent studies determined how lowering the protein content of diet would affect growth performance of shrimp reared in biofloc conditions. In one of the study it was found that at least 10% of protein content in feed can be reduced when *F. paulensis* postlarvae are cultured in BFT conditions. It was observed that shrimp fed with less than 25% crude protein under biofloc conditions performed similarly to shrimp raised under regular clear-water intensive culture with a 37%-protein diet. The biofloc system also delivered better consistent survival rates, especially at higher density. A low-protein biofloc meal-based pellet (25% CP) was evaluated as a replacement of conventional high-protein fishmeal diet (40% CP) for *L. vannamei* under biofloc conditions. The results showed that is possible to replace 1/3 part of a conventional diet by alternative low-protein biofloc meal pellet without interfering survival and shrimp performance.

Our experimental results with graded levels of protein in a biofloc system have indicated that the dietary protein level could be reduced to 32% without reduction of performance compared to 40% dietary protein level.

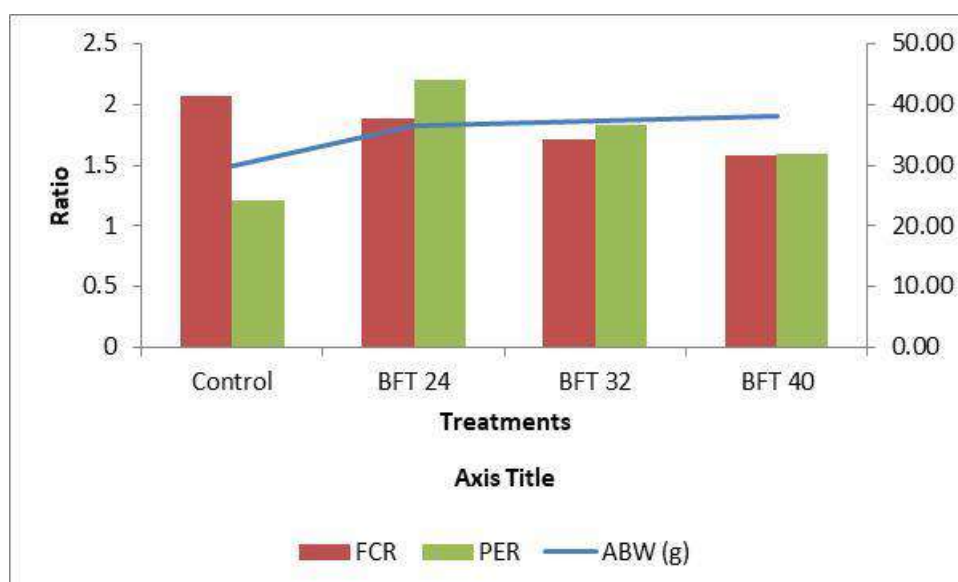


Fig. 2. Role of Biofloc in replacing fishmeal in the feed

The major limiting factor for aquaculture growth will be the availability of fishmeal and fish oil for aquafeed production in the years to come. Hence the fish nutritionists are trying

to find alternatives to the Fishmeal and fish oil. This inspired researchers to develop the biofloc technology, which is also applicable to intensive and semi-intensive systems. With biofloc technology, where nitrogenous waste generated by the cultivated organisms is converted into bacterial biomass (containing protein), *in situ* feed production is stimulated through the addition of an external carbon source. Although bioflocs show an adequate protein, lipid, carbohydrate and ash content for use as an aquaculture feed, more research is needed on their amino acid and fatty acid composition. Now, fishmeal and fish oil supply essential amino acids (such as lysine and methionine) that are deficient in plant proteins and fatty acids (eicosapentanoic acid and docosahexanoic acid) not found in vegetable oils. Although bioflocs meet nutritional standards to serve as a aquaculture feed in general, research has shown that the capacity of the technique to control the water quality in the culture system and the nutritional properties of the flocs are influenced by the type of carbon source used to produce the flocs. Different organic carbon sources each stimulated specific bacteria, protozoa and algae, and hence influenced the microbial composition and community organization of the bioflocs and thereby also their nutritional properties. Feeding experiments revealed that besides these characteristics, the type of carbon source also influenced the availability, palatability and digestibility for the cultured organisms. However, further research should focus on the use of low-cost non-conventional agro-industrial residues as carbon source and hence upgrade waste to nutritious feed. Different carbon sources will stimulate the growth of the indigenous microbiota in another way and thus exert a distinctive effect on water quality, *in situ* feed production and utilization of the flocs by the cultured organisms. In addition, not only the carbon source, but also the indigenous microbiota present in the pond water will put forth a characteristic effect that needs to be considered. An important factor here is to determine the role of algae and their interaction with the bacteria in the bioflocs.

An interesting topic for further research could be the identification of micro-organisms (bacteria and micro-algae) that are able to produce bioflocs with the desired nutritional properties and a good ability to control the water quality. Such micro-organisms could be used as an inoculum for the start-up of aquaculture systems with biofloc technology. All these findings and possible *modus operandi* emphasize the need for further study of biofloc composition in order to achieve a desired nutritional outcome, since different research groups have obtained different results in respect to biofloc nutritional composition.

The BFT also helped in replacing fish meal up to 40% in shrimp diets. In addition efforts were also made to use biofloc meal as the feed ingredient in shrimp feeds by replacing fish meal. In a trial performed in clear-water conditions detected that fishmeal can be completely replace with soy protein concentrate and biofloc meal (obtained from super-intensive shrimp farm effluent) in 38% CP diets without adverse effects on *L. vannamei* performance. Moreover, observed that biofloc produced in bioreactors using tilapia effluent and sugar as a growth media could offer an alternative protein source to shrimp feeds.

Microbial floc-based diets significantly outperformed control fishmeal-based diets in terms of weight gain per week with no differences in survival.

Role of Biofloc on Broodstock Nutrition

The BFT has been successfully applied for grow-out, but the information little is known about biofloc benefits on breeding. The nutritional problems of brood stock and biosecurity issues were remain unresolved and alternatives should be evaluated for broodstock. Breeders raised in BFT limited or zero water exchange system are nutritionally benefited by the natural productivity (biofloc) available continuous *in situ* nutrition during the whole life-cycle. Biofloc in a form of rich-lipid-protein source could be utilized for initial stages of broodstock's gonads formation and ovary development. The continuous availability of nutrients especially fatty acids which are not oxidized could promote high nutrient storage in hepatopancreas, transferred to hemolymph and directed to ovary, resulting in a better sexual tissue formation and reproduction activity. Furthermore, production of broodstock in BFT could be located in small areas close to hatchery facilities, preventing spread of diseases caused by shrimp transportation. In conventional systems breeders used to be raised in large ponds at low density. However, risks associated with accumulation of organic matter, cyanobacteria blooms and fluctuations of some water quality parameters (such as temperature, DO, pH and N-compounds) remains high and could affect the shrimp health in outdoor facilities. Once the system is stable BFT provides stabilized parameters of water quality when performed in indoor facilities such as greenhouses, guaranteeing shrimp health.

NUTRITIONALLY RICH BIOFLOC MEAL COULD BE A SUSTAINABLE INGREDIENT IN AQUA FEED FORMULATIONS

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The bio-flocs are consortium of particulate matter formed predominantly by a biota of aerobic and heterotrophic bacteria, protozoa, microalgae, metazoan, exoskeletons, feces and remains of dead organisms. It has been found that, bacteria and some microalgae produces exopolysaccharides (EPS) such as uronic or pyruvic acid under stressed condition, and these polymers are responsible for the adhesion and aggregation of dispersed cells of bacteria and microalgae, called bioflocs. These flocculated microorganisms will be denser and tend to settle down at the bottom. Continuing research on biofloc around the world has evidenced the manifold direct and indirect benefits for the farmed species, environment and production economics. Though the production and management of this multi-trophic microorganisms is a complex process, by having engineer designed rearing system and adopting smart management practices biofloc will be simply achievable.

Aquaculture is growing fast and contributes about 50% to the global total fish production, and expected to grow at much faster rate to supply the increasing demand of fish for human consumption (FAO, 2016). Fish culture involving formulated feed usage is increasing in geometric proportion, and it is creating huge pressure on ingredient supply, especially fishmeal. Therefore, finding sustainable alternate feed ingredients is the biggest challenge for the aqua nutritionist. We expect biofloc meal could be a sustainable ingredient for future.

Significance of biofloc in shrimp and fish nutrition:

Providing good water quality through biological processing involving consortium of bacteria and microalgae ('BIOFLOC') and use of bioflocs as a fresh feed (in situ feeding) within the system is considered to be a low cost sustainable feeding tactic, and is receiving popularity in farming of white leg shrimp around the world. Biofloc is found to be a nutritionally rich and balanced with good amount of protein, minerals and other micronutrients. More importantly, it is interesting to note that bioflocs or its attached microorganisms could exert a positive effect on the digestive enzyme activity of shrimp or fish. Bioflocs not only act as a feed, but also manages the water quality with no any additional cost. Even though bioflocs are known to be consumed a lot by the white leg shrimp as fresh feed, it may not be consuming all the available biofloc biomass, and many will be discarded during water exchange. Furthermore, other cultured species of shrimp and fish are either physiologically or anatomically restricted to show that much attraction towards consumption biofloc. In such situations, most of the floc biomass will

be thrown in to the receiving water body. Here there is an immense scope for harvesting these nutrient dense feed particles with advent of various filtration and separation technologies, and further processing it could add a potential feed ingredient to the aquafeed ingredient basket.

Concept of biofloc formation and biofloc meal production as a sustainable approach:

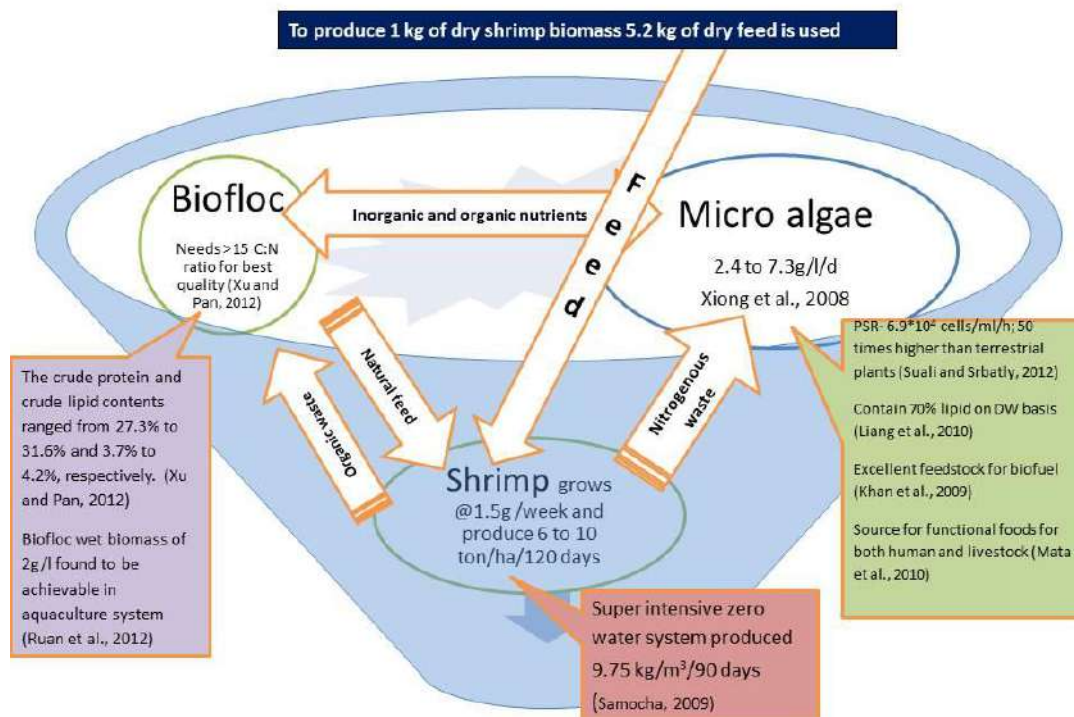
Feed is a major input in any shrimp farm and it never been completely utilized by the shrimp for its growth. Naturally, complete utilization of feed consumed by any animal is impossible, and shrimp is not an exception. Considering a global average of FCR 1.4, one can calculate that, to produce 1kg fresh shrimp (with 80% water) we need 1.4kg dry feed (with 10% water). In equivalent figures of dry matter, this will be like this; to produce 1 kg of dry shrimp biomass we need 6.3 kg of organic matter. Eliminating shrimp biomass (1 kg), about 5.3 kg of organic matter is thrown as waste either on the pond bottom or in to the nearby water body during water exchange. However, under existing practices, even in zero water exchange systems, except the nutrients in the shrimp biomass, nothing is recovered back. Instead, nutrients are simply thrown as sludge or along with the discharged water. The concept of having biofloc to recycle both the organic and inorganic nutrients by utilizing the services of microalgae and bacteria, and providing biofloc itself as a natural feed for the shrimps is a novel concept, and it would be eco-friendly, cost-effective, and having a lot of potential for further intensification by increasing the shrimp stocking density in a unit area provided. On the other hand, shrimp being an aquatic animal which passes water through their gill lamellae for oxygen uptake from water, sustaining plenty of suspended particles in the ambient water may lead to gill congestion (Xu et al., 2012). Therefore, it is assumed that constant removal of flocculated biomass from the system would be favourable for shrimp. Furthermore, it also makes space for fresh algal and bacterial cells to grow, which in turn increase the productivity of the system. The removed suspended particles may be concentrated, dewatered and dried to produce nutrient rich biofloc meal. This is not only a nutritious meal, but also a sustainable ingredient from the aquaculture practice itself.

Biofloc and biofloc meal:

Biofloc is a clumpy assemblage of microorganisms (bacteria, micro algae, cyanobacteria, fungi, protozoans, micro-zooplanktons, etc.) with dead particulate organic matter suspended in the water column of an intensively aerated or agitated aquaculture system. Research showed that, bacteria and some microalgae produces exopolysaccharides (EPS) such as uronic or pyruvic acid (Shipin et al. 1999) under stressed condition. These polymers act as adhesives to aggregate the dispersed cells of bacteria, microalgae and other particulate organic matter to form a clumpy mass called bioflocs (Frølund et al. 1996). These dense flocculated assemblages will tend to settle down at the bottom of rearing system.

Biofloc meal is nothing but a concentrated, dewatered and dried form of biofloc harvested from the system. Harvesting biofloc would be a costly process, which may add cost to the product. It needs to be simplified in an economical way. While the existing processes are scaled up to a larger scale, the price can be significantly reduced. The drying can be either spray drying or drum drying. Spray drying will preserve the nutritional value of the meal. Freeze-

drying would be an option for keeping the nutrients of the biofloc meal intact, and the meal can be used as ingredient in speciality feeds such as brood stock and larval feeds.

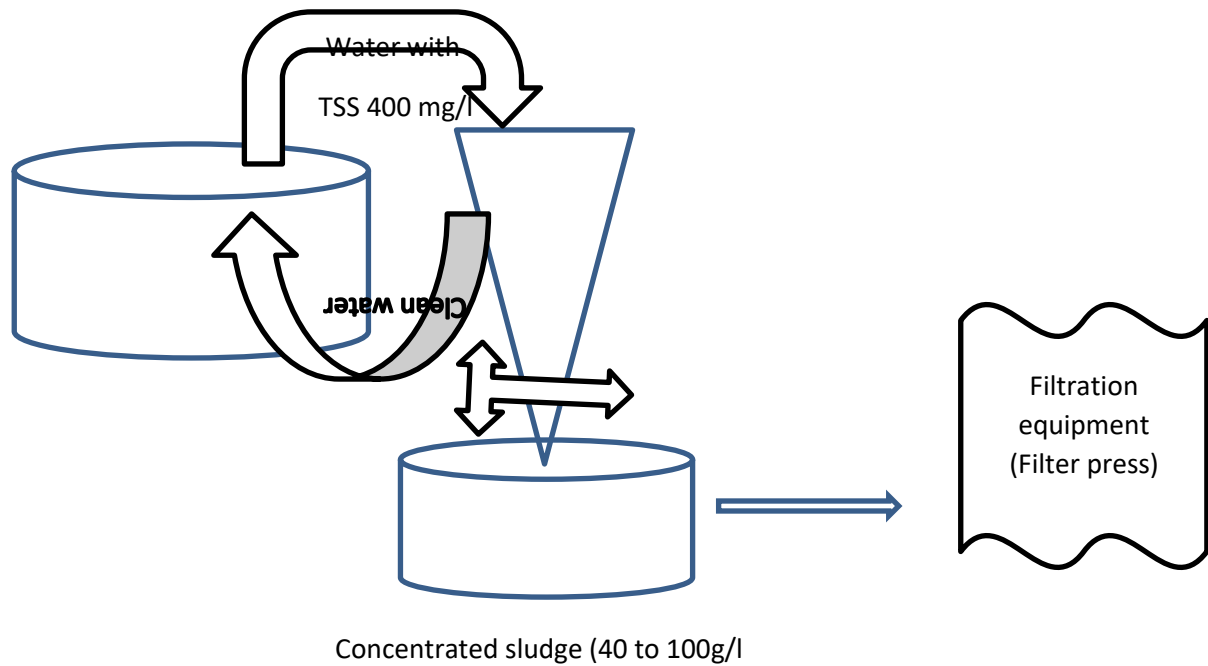


Culturing and harvesting of Biofloc

Self-circulating out-door microcosm tanks were used for producing bioflocs. Bioflocs were produced against various C:N ratios and presence of fish and shrimp. Radial flow settlers in combination with filter meshes were used for harvesting the biofloc from the water. C:N ratios were adjusted using formulated feed and jaggery.



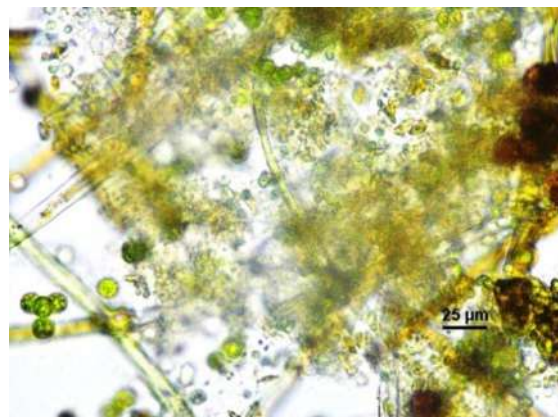
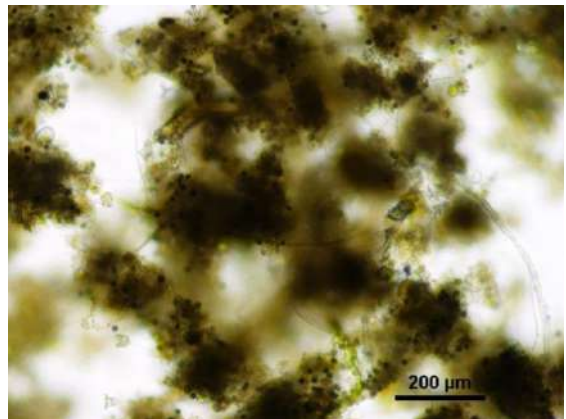
Fig: Self-circulating out-door microcosm tanks used for producing bioflocs



Schematic diagram explaining the harvesting process of biofloc from the culture tank



Harvesting of bioflocs using radial settler



Nutritional value of biofloc meal:

Since biofloc is formed of various groups of microorganisms and organic particulate matter by recycling the dissolved nutrients from feed, naturally it will become nutrient dense material. Reported protein and lipid content of biofloc collected from different culture conditions ranges from 25 to 50% and 0.5 to 15% respectively (Khun et al., 2009). Bioflocs were found to be good source of nutrients like protein, lipids, vitamins and micro nutrients. Bioflocs may also have potential to provide exogenous enzymes to the cultured animal and some probiotic effects. There are differing judgment about the adequacy of bioflocs to provide the limiting amino acids methionine and lysine.

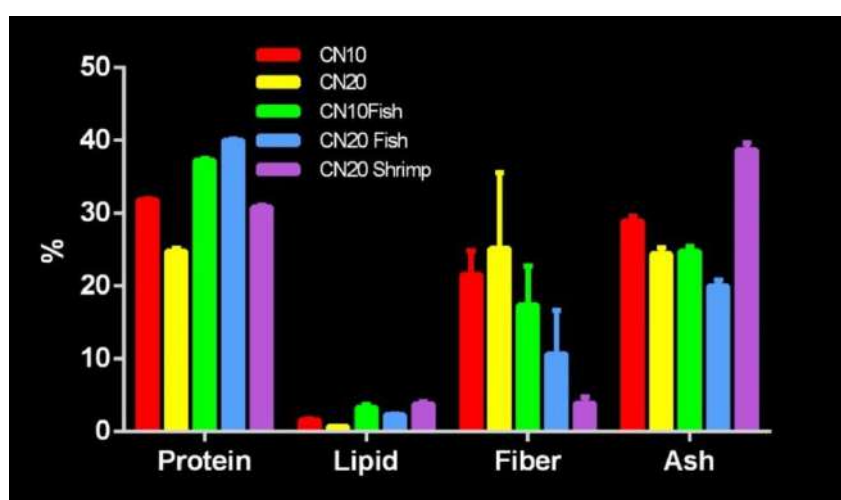


Fig: Nutritional characteristics of the biofloc produced under varying C:N ratio

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CURRENT STATUS OF AQUA FEED INDUSTRY IN INDIA AND CIBA'S INITIATIVE IN NEWER FEED TECHNOLOGIES

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Aquaculture has emerged as one of the fastest growing food producing sector in the current decade. It is estimated that global fish production would increase by 19% between 2014 and 2024 with the main driver being formulated feed. India ranks second in aquaculture production. Indian aquaculture is mainly contributed by freshwater aquaculture while the brackishwater aquaculture is poised for vertical and horizontal expansion during the years to come. The rapid expansion of aquaculture has to be supported by the availability of cost effective sustainable technologies.

Aqua feeds in India have evolved commercially in late 90's for shrimp culture and then for fishes. In the brackishwater aquaculture sector majority of the feed used is scientifically formulated compounded feed produced by multinational / Indian company. There are some small players with a capacity to produce 1 tonne per hour operating to cater to the need of local area/undertaking job works for the nearby farmers. In the fresh water aqua feeds traditional feeding using farm made feed comprising rice bran and locally available plant protein source is the predominant feeding system where in about 80-85% of the potential is unexplored for manufactured feeds. The sinking feed for carps and the extruded floating feeds for fish are making a steady and positive impact in changing traditional feeding system with the focus on increasing the productivity on a sustainable manner in the longer run.

Brackishwater aquaculture is synonymous with shrimp aquaculture in India and tiger shrimp, *Penaeus monodon* is the main species cultured in the brackishwater till 2010. During the year 2011-12, a total of 135778 MT of tiger shrimps were produced from an area of 115342. After the introduction of the pacific white shrimp in 2009 there is steady and sharp increase in culture area as well as production and *Litopenaeus vannamei* has become the main species cultured in brackishwater. The growth of *L.vannamei* production has been stupendous. From a production of about 18247 MT from 2930 ha in 2010-11, the *L.vannamei* production reached 353413 MT in 2014-15 registering an increase of 1837% in production. The boom period for vannamei farming began in the late 2011, when shrimp prices were very good, and the positive trend continued till early 2014. However, due to recurrent problem in diseases such as white spot virus and running mortality syndrome there is a sudden decline production of farmed shrimp. This decreased production coupled with

spiraling hike in all input prices particularly the feed prices has made a significant impact on the profitability of the shrimp farming. The cost of production of shrimp has increased from Rs. 180 per kg of shrimp in the year 2010 to Rs. 280 per kg. This sharp increase in price is mainly due to the steady increase in the cost of feed. The feed cost per kilo of shrimp produced alone reached an all-time high of Rs. 135 per kg. This scenario resulted in considerable difficulties for the small and medium scale farmers as they could not cope up with the double blow of increased feed price and a drop in shrimp price.

This increase in price has not been commensurate with farm gate price offered to the farmer. This has made serious dent in the profit margin and farmers started looking for ways and means to reduce the cost of production of shrimp. The feed cost has been considered as one of the major factor for the increased cost of production and the feed cost is mainly determined by its protein content. In this context, it is pertinent to note that *L. vannamei* is an omnivorous scavenger and is less aggressive and less carnivorous than *P. monodon*. The available research information on the nutritional requirement of this species also indicates that it requires less protein than the tiger shrimp. Further, it can also accommodate more plant protein sources in the diet than the tiger shrimp. These inherent advantages should have resulted in availability of more cost effective feeds. However, the analysis of actual scenario revealed an interesting picture. All the major feed companies in India maintained their protein content for tiger shrimp in the range of 38-40% while the crude protein content in vannamei feed was kept in the range of 34-36%. The average price of shrimp feed for both the species during the period from 2010 to 2015 is given below.

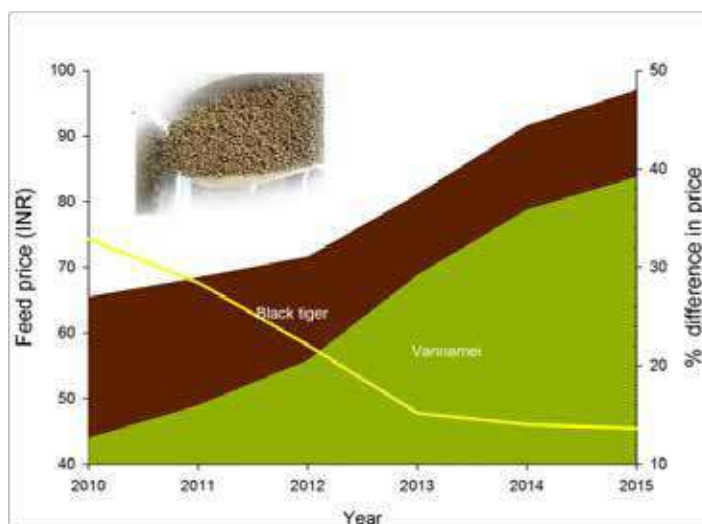


Fig1. Average price of shrimp feed

During the year 2010, the cost of vannamei feed was Rs. 44 per kg against Rs. 66 per kg for the tiger shrimp feed and the price difference between these two feeds was Rs 22 per kg. Even though the nutrient contents were maintained uniformly over the years the price difference between these feeds showed a consistent decrease. Currently, the cost of

vannamei feed has reached its peak at Rs.85per kg while the tiger shrimp feed costs about Rs. 98 per kg and the price difference narrowed down to Rs. 13 per kg. This showed that the cost of vannamei feed available to the farmer at present is high against its nutrient content and the feed cost has to be brought down to make the vannamei farming more profitable and affordable by the shrimp farmer.

As per the available information, during 2014 a total of 1.25 million metric tons of aqua feed has been produced in India comprising of about 600,000 tons of shrimp feed and 650,000 tons of fish feed. Out of the total shrimp feed produced vannamei contributed about 80-85%. The total shrimp feed is produced by the eight major feed mills and 30 small feedmills.

Availability of cost effective feed is the felt need of the farmers at present and they started exploring the possibilities of producing their own feed inorder to reduce the cost of production. Many bigfarmers and farmer's group/cooperatives are contemplating to establish smaller feed mill to produce feed for their own requirement. In addition they can also cater to the small farmers located in their region with the availability of cost effective feed. This direct marketing channel from the feed mill to the farmer would result in availability of cost effective feed. Central Institute of Brackishwater Aquaculture (CIBA) has developed the indigenous shrimp feed technology and helping them in such endeavors. During the year 2015, CIBA has entered into MOU for feed technology transfer with four such entrepreneurs. Feed mill with a production capacity of 1-2 tons per hour is being established in the state of Gujarat, Andhra Pradesh, Kerala and West Bengal in India. There is a great scope for establishing many such feed mills in the coastal states of India. This would result in availability of cost effective feed for small and medium farmer. Brackishwater aquaculture technology is evolving with an array of new array of feed technologies and farming system and the details are given below.

Indigenous Shrimp Feed Technology

CIBA has taken up research program on a mission mode approach and has made rapid strides in the area of shrimp feed development. Knowledge on nutritional requirements of candidate species is essential for the development of feed and hence to begin with the nutritional requirement of the shrimp species viz., *P. monodon* and *P. indicus* have been extensively researched upon.

The feed technology was developed through formulation of several test feeds using indigenous ingredients to meet the dietary requirements of tiger shrimp (*P.monodon*) and Indian white shrimp (*P.indicus*). These test feeds were first evaluated in laboratory experiments and then in yard experiments on three size groups namely 2-5g, 5-10g and 15-25g for developing three grades of feed namely Starter, Grower and Finisher. The feed formulations which gave best growth and feed conversion ratio (FCR) were selected for up-

scaling. A pilot-scale Ring-Die pellet feed mill of capacity 500kg per hour was set up at the Muttukkadu experimental station of CIBA. The pellet mill was integrated with a hammer mill, micro-pulverizer, sieve assembly, ribbon mixer, steam boiler and crumbler for completing the feed processing. All the machinery is of indigenous origin.

The multi locational demonstration of indigenous shrimp feed technology resulted in commercialization of CIBA developed shrimp feed technology to the following firms.

- M/S Bismi Feeds (P) Ltd, Deencomplex, OSMNagar, Mayiladuthurai, Nagapattinam District, Tamil Nadu
- M/S. Pisciculture care Unit, Village Madhubati, P.O. Kamarpukr, Hooghly District, West Bengal The feed produced by M/S Bismi feeds using CIBA shrimp feed technology has gained popularity among the farmers in Tamil Nadu and has become the sought after brand. The feed produced using CIBA shrimp feed technology is being sold @ Rs 4-6 lower than the reputed commercial brand shrimp feeds. The reduced cost of shrimp feed is due to the use of indigenous raw materials and machineries.

Feed for vannamei: *Vannamei* ^{Plus}

CIBA has developed a cost effective grow out feed using indigenous ingredients. This feed has been tested in a farmer pond at Gujarat and compared against the commonly used commercial shrimp feed. The cost of the commercial feed available to the farmer was about Rs.78 per Kg while the cost of feed (inclusive of cost of ingredients + processing and transportation cost) to the farmer was Rs. 54 per kg. At the end of 117 days of culture the results showed that shrimps have attained an average final body weight of 27.1g in the pond fed with CIBA feed while the control pond shrimps have attained the final body weight of about 24.6g. The total quantity of shrimp harvested was about 2017 kg in the CIBA feed fed pond while it was about 1913 kg in the control pond fed with commercial feed. The FCR obtained was 1.68 with CIBA feed and 1.79 in the commercial feed. Interestingly the feed cost to produce one kg shrimp was Rs.91 with CIBA feed against Rs.140 in the commercial feed. This could be potential saving for the shrimp farmers and would pave the way for considerable reduction in production cost with better profitability for small and medium farmers. These results showed that the indigenous *vannamei* grow- out feed developed by CIBA could be cost-effective and important substitute to bring down the cost of production and increase the profitability of Indian shrimp farmers.

This feed technology has been commercialized to the following firms

1. Sai AquaFeeds
2. Poshak Bio Research PvtLtd
3. BTrevelations

4. M/S. M.KFeeds
5. Kavya Aquafarm
6. West land marine PvtLtd.

Specialty feeds

The nutritional requirement of shrimp varies depending on the environment in which it is cultured. In India the shrimp farming is being practiced from low saline (1to5ppt) to high saline conditions (35- 45ppt). Considering these specific dietary requirements for improving the growth and FCR under high and low salinity, trials have been carried out and feeds for low and high saline conditions have been developed. These feeds are ready for field testing and commercialization. The other specialty feeds developed were to address stress and attractability. Under intensive / semi intensive farming, shrimps are subjected to considerable stress which in turn affects the growth and FCR. To address this, various stress busters have been explored and feed with specific additives to mitigate the stress have been developed. Extensive research efforts on palatability and attractability lead to the development of cost effective feed with good attractability and palatability.

Low protein and Low fish meal based feeds

Fish meal is the mainstay in shrimp feed and CIBA has initiated major research programme on replacement of fishmeal with alternate protein sources with reasonable rate of success. This has led to development of organic shrimp feed wherein, there restriction on the usage of fish meal and fish oil. Similarly, there are exclusive feeds for feeding the shrimp under biofloc system. Since the biofloc contributes considerable amount of micronutrients it is apt to reduce the protein content and use more of plant protein based ingredients to meet the protein requirement. This would help in reducing the cost of feed and will pave the way for sustainability.

Broodstock Feed Etro brood^{Plus}

Pearlspot is a popular aquarium and food fish, promoted as potential candidate for brackishwater farming. Farmers produce pearlspot seeds in large ponds in traditional manner by feeding the adults with commercial carp feeds and farm made feeds. Seed production with such a low fecund fish in this manner is labour intensive, less productive and uneconomical. CIBA developed a broodstock diet (Etro brood^{Plus}) to provide specific nutrients vital for maturation and spawning, and demonstrated for the first time that pearlspot can be made to spawn more than 4 times/year (average) in 1000 L tanks with an average fry yield > 2200/spawning. CIBA also developed a larval feed and standardized a feeding strategy to rear the pearlspot larvae with maximum survival and fast growth in the absence of parental care.

MILKFISH BROOD^{Plus} and MULLET BROOD^{Plus}

Institute has developed a unique formulation for Milkfish Broodstock using novel and speciality ingredients and the feed has been found to be effective for Milkfish breeding and this has been tested and validated. The feed developed is also being used for Milkfish Broodstock by Aditya Fish Hatcheries under the Collaborative research cum technology transfer program.

Shrimp Larvi^{Plus} : Shrimp Larval Feed

Development of inert diet for rearing shrimp larvae has been a challenging task. This is mainly because, besides providing a balanced nutrition package in a tiny particle, which should be digested and assimilated, the diet particles should be designed to keep them suspended in the rearing medium so that the filter feeding larvae can utilize it. Live food organisms such as brine shrimp nauplii are still indispensable for rearing shrimp larvae. Nevertheless, artificial diets are also supplemented to overcome the uncertainties associated with mass production of live food organisms, their nutritional imbalances and cost. Increasing costs of imported brine shrimp cyst have pushed the cost of production of shrimp seed. With fluctuating international market for shrimp the farmers are looking for reduction in input costs in shrimp production. Use of inert diet supplements for feeding shrimp larvae would certainly reduce the cost of production of shrimp seed. *Shrimp Larvi^{Plus}* has been successfully developed for feeding the larvae and post larvae of shrimp. It has been tested in vannamei larvae in commercial hatcheries and the technology is being commercialized with M/S. MARITECH.

Seabass^{Plus} : Feed for seabass

Seabass^{Plus} is a formulated pellet feed developed by CIBA for the nursery and grow-out culture of Asian seabass, *Lates calcarifer* and contains 40-50% protein. It meets the dietary requirements of Asian seabass for optimum growth with good feed efficiency. CIBA *Seabass^{Plus}* uses indigenously available raw materials. The feed processing technology for different grades was standardized in a Ring-Die pellet mill and also in a Twin-Screw Extruder at the Pilot-scale feed mill of CIBA at Muttukadu. The feeds were systematically developed through formulation of several feeds using selected indigenous feed ingredients and evaluation of the test feeds in laboratory and yard trials and selecting the best performing formulation. Using the selected formulation, the feed was processed into granules and pellets with indigenous feed mill and processing technology to suit the growing stages of Asian seabass in nursery and grow-out systems. The feeds were extensively tested in the institute facilities and also in selected farmers' fields in different states such as Andhra Pradesh, Maharashtra and Tamil Nadu. CIBA Bhetki ahara resulted in excellent growth and survival of fry in nursery and grow-out systems in these field trials. The fish was successfully grown to 1 kg in 8-9 months with an impressive FCR of 1.5 to 1.8.

It is believed that the performance and cost effectiveness of this indigenous feed will go a long way in propagating large scale farming of Asian seabass.

Seabass Larvi^{Plus} : Feed for seabass larva developed and tested in commercial Hatcheries

Feed for Crab : Scylla^{Plus}

Similar to the feed development programme of shrimp and fish considerable research has been carried out in the development of feed for mudcrab. In this context feed for fattening and grow out culture has been developed and field tested with highly encouraging results.

CIBA developed low cost indigenous automatic feeder for *L. vannamei*

Automatic feeders are more suitable for *L. vannamei* culture due to its feeding behaviour as it is a column dweller and it needs continuous feeding. Entrepreneur farmers have imported automatic feeders and it is costly. These prompted CIBA to design and develop a low cost feeder for the use for small farmers. The design of an automatic feeder developed at CIBA consist of four major components, a feed hopper, a mechanisms for feed distribution an electrical power supply/ solar power and a control unit for operating (frequency and time of distribution). The hopper bottom has been designed such that the angle of repose of the hopper bottom is greater than the angle of repose of the materials for easy dispensing from the hopper. Motor unit was fixed with variable speed regulator to control the dispensing of the feed. Two timers with digital display were set such that the dispensing of the feed and the duration of feeding could be adjusted easily by the farmers. This has been successfully demonstrated in the shrimp farm at Marakkanam, Tamil Nadu. The cost is only Rs 15,000/- and it is easily operated and adjustable with digital display. The provision for incorporation of solar energy using DC motor has been done. Trial demonstration showed that the radius of dispersion is 26 m and quantity of feed can be adjusted as per the need.

Conclusion

The feed development programme at CIBA matched the pace and growth of aquaculture industry. The research planning in the areas of aquaculture nutrition are in tandem with the priority of the farming community and have lead to development of indigenous feed and processing technology for brackishwater shrimp and fish. The successful commercialization of CIBA shrimp feed technology and its presence in the market is the testimony to the strength of feed development programme of this institute.

EMERGING DISEASES IN BRACKISHWATER AQUACULTURE IN INDIA

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Introduction

Aquaculture sector is expanding at a rapid pace to achieve the goal of nutritional security. The Indian aquaculture production has considerably improved during the last several years particularly to mention about the introduction of the exotic Pacific white shrimp, *Litopenaeus vannamei* and the subsequent success in achieving higher production. Availability of imported specific pathogen free (SPF) broodstock provided the much needed boost to India's brackishwater aquaculture sector. Because of its SPF status, fast growth rate and culture feasibility in wide salinity range, this got readily accepted by the farmers and has become the dominant cultured species at present. The shrimp production through aquaculture touched an all-time high of 815,745 metric tons during 2020-2021, contributing to export revenues to the tune of US\$4.5 billion. Intensification of shrimp farming has exacerbated the epizootics and disease issues are becoming a major constraint, affecting productions and profitability. Among the OIE (World Organization for Animal Health) listed viruses of the farmed crustaceans, the white spot syndrome virus (WSSV) and infectious hypodermal hematopoietic necrosis virus (IHHNV) have been frequently reported from Indian subcontinent and both these pathogens can be considered as endemic in India. During the recent times, infectious my necrosis virus (IMNV) has also been detected from several parts of the country causing mortality to different extents. Other viral pathogens such as, Taura syndrome virus (TSV), yellow head virus (YHV) and the recently emerged Decapod Iridescent virus 1 (DIV 1) which have been otherwise responsible for causing losses to aquaculture in the Americas and the Southeast Asian countries have so far not been reported from India. Similarly, the early mortality syndrome (EMS) or acute hepatopancreatic necrosis disease (AHPND) in the South East Asian countries has also been found to have severe economic impact on shrimp industry of these regions. During the last several years, a number of syndromes such as running mortality syndrome (RMS) or chronic mortality syndrome (CMS), white faeces syndrome (WFS)/ white gut syndrome and stunted growth / growth retardation have been negatively affecting shrimp aquaculture in India. Some of these problems associated with current shrimp culture practice in India are discussed in this article.

Infectious Myonecrosis Virus (IMNV)

Amongst the viruses, IMNV is considered as an emerging virus for shrimp culture practice in India. Otherwise, this virus is also limited only to some of the countries where it has been reported to cause economic loss. Based on some published reports, IMNV can bring severe economic losses to penaeid shrimp aquaculture.

This virus was first reported in shrimp farms of Brazil in 2002 (Nunes et al., 2004). Similarly, the first outbreak in Asia-Pacific region was reported from Indonesia in 2006 (Senapin et al., 2011). Subsequent to this the virus has also been reported in some of the Asian countries

(OIE, 2019; Tang et al., 2019). Based on the recent investigation, isolates of IMNV representing new genotypes have been reported to be associated with recent disease outbreaks in *P. vannamei* shrimp farms in Indonesia (Mai et al., 2019).

Focal to extensive white necrotic areas in striated (skeletal) muscles, especially in the distal abdominal segments and tail fan and redness in some affected shrimp are some of the focal disease signs. All the life stages of shrimp including post larva, juveniles and adult stages have been reported to get infected by this virus.

Shrimp farms infected with IMNV shows cumulative mortality ranging from 40% to 70% in juveniles and adults (Nunes et al., 2004; Poulos et al., 2006). Though the disease was restricted only to some of the countries like Brazil in South America (Lightner et al., 2004; Melena et al., 2012) and Indonesia in Asia (Mai et al., 2019; Senapin et al., 2007; Taukhid & Nuraini, 2009), this has also been detected in India. Occurrence of disease and the subsequent economic losses caused by this exotic disease in northern parts of India has been (Hameed et al., 2017). Subsequently, this was also reported from the southern part of India (Jithendran et al., 2021).

Hepatopancreatic microsporidiosis

Hepatopancreatic microsporidiosis (HPM) is caused by *Enterocytozoon hepatopenaei* (EHP), an yeast-like fungus belonging to a group called “microsporidia”. It was first reported in 2004 as an unnamed microsporidian from growth retarded black tiger shrimp *Penaeus monodon* from Thailand. There are no other specific signs and symptoms of EHP infection in shrimp. It was discovered in slow growing shrimp and also from shrimp that exhibited mortality associated with white faeces syndrome. The target organ of EHP is hepatopancreas and affects its digestive and absorptive functioning resulting in poor growth and immunity. Although EHP does not appear to cause mortality in *P. monodon* and *L. vannamei*, information from shrimp farmers indicates that it is associated with severe growth retardation in *P. vannamei*. Estimated loss of USD 232 million per annum due to EHP alone was reported from Thailand (Shinn et al., 2018). In India, the production loss of 0.77 million tonnes corresponds to an economic loss of USD 567.62 million per annum has recently been reported (Patil et al., 2021).

EHP produces smaller spores of approximately 1 µm in length can be detected microscopically and by polymerase chain reaction technique. Diagnosis of infection can be achieved by microscopic examination of squash preparation of hepatopancreas and demonstration of the spores. Other diagnostic methods such as histopathology, *insitu* hybridization, polymerase chain reaction (PCR), loop-mediated isothermal amplification (LAMP) and real-time PCR have also been reported.

It has been found that EHP can be transmitted directly from shrimp to shrimp by cannibalism and cohabitation. *E. hepatopenaei* (EHP) is confined to hepatopancreatic (HP) tubule epithelial cells of the shrimp and shows no gross signs of disease. It is likely that other penaeid shrimp and crustaceans may also be susceptible to infection. The EHP spores in fecal pellets or dried cadavers can remain viable up to six months and retain infectivity for over a year under aqueous conditions.

EHP can be found as co-infection with WSSV, HPV and IMNV. Experimental evidence indicates that it can also have co-infection with AHPND causing bacteria whereas natural infection shows presence with *Vibrio parahaemolyticus*. It is predicted that such types of co-infection can increase the disease precipitation causing more harm to the host.

Shrimp farmers and hatchery operators should monitor *L. vannamei* and *P. mondon* for EHP in broodstock, PL and rearing ponds. The best approach for maturation and hatchery facilities to avoid EHP is not to use wild, captured, live animals (Eg., live polychaetes, clams, oysters, etc.) as feeds for broodstock. This is because, it has recently been reported that polychaete can be a carrier for EHP. Better would be pasteurization (heating at 70°C for 10 minutes). Another alternative would be to use gamma irradiation with frozen feeds. Alternatively, polychaetes could be selected and tested for freedom from shrimp pathogens and then reared as broodstock feed in biosecure settings designed to maintain their freedom from shrimp pathogens (*i.e.*, SPF polychaetes). There is no drug for the control of EHP infection in shrimp. Application of lime and maintaining the soil pH to 12 has been suggested for the disinfection of ponds. As per one of the recent reports, chlorine was found to effectively destroy EHP spores and the same can be applied in the farms before the start of the culture. Better management practices (BMPs) and proper biosecurity is the only way to keep this parasite away from the aquaculture ecosystem.

White faeces syndrome

White faeces syndrome (WFS) reported since last decade, has recently been noted as serious problem for *P. vannamei*. It has been estimated that the Thai production losses due to WFS in 2010 were 10–15%. WFS has been reported from both cultured black tiger shrimp and pacific white shrimp. It usually occurs after about 35–45 days of culture (DOC) and it may be accompanied by shrimp mortality. Ponds affected with WFS show white faecal strings floating on the pond surface while the shrimps show white/golden brown intestine, reduced feed consumption, growth retardation and often associated with looseshell. The disease can cause moderate to severe economic loss by reducing the shrimp survival by 20–30 percent. While investigating the aetiology of WFS, it has been observed that it is associated with the presence of vermiform gregarine like bodies, Vibriosis, *Enterocytozoon hepatopenaei*, blue green algae and loose shell syndrome. Sriurairatana et al (2014) revealed that 96% of the ponds exhibiting WFS presented vermiform bodies resembling gregarines. When the contents of the gut or faecal strings were examined in squash mounts with the light microscope, they consisted of masses of vermiform bodies that superficially resembled gregarines. Bacteriological results showed that total bacteria and *Vibrio* spp. found in haemolymph and intestine were significantly higher in diseased shrimp than in healthy shrimp. Six species of fungi (*Aspergillus flavus*, *A. ochraceus*, *A. japonicus*, *Penicillium* spp., *Fusarium* spp., and *Cladosporium cladosporioides*) were isolated from shrimp naturally infected with white faeces syndrome. Histopathological examination revealed diffused haemocyte encapsulation and dilated hepatopancreatic tubules accompanied by necrosis. Tangprasittipap *et al.*, 2013 reported that the microsporidian found in *L. vannamei* is nonspecific with previously described *E. hepatopenaei* and it is not causally associated with WFS. Sriurairatana et al. (2014) based

on transmission electron microscopic study suggested that vermiform structures superficially resembling gregarines and commonly found in the shrimp hepatopancreas (HP) are not independent organisms but result from the transformation, sloughing and aggregation of microvilli from the HP tubule epithelial cells themselves. The denuded epithelial cells subsequently undergo lysis and possibly lead to the phenomenon called white faeces syndrome (WFS). The cause of white faeces syndrome and treatment is uncertain. However, reduced stocking density, proper water exchange together with better management practices will be helpful in evading White Faeces syndrome (WFS).

Running Mortality Syndrome (RMS)

Since 2011, a new syndrome, loosely termed by the farming community as running mortality syndrome (RMS) or chronic mortality syndrome (CMS) has been causing substantial morbidity and mortality affecting shrimp farming. The affected ponds show different mortality patterns with unusual symptoms, and no relation to any known pathogens associated with slow mortality rate (e.g. <1%/day), but the cumulative loss over the period will be significant. Some farmers have lost up to four crops, with mortalities reaching 70%. Affected shrimp often show white abdominal patches and generally mortalities start after a month or 40 DOC, but a portion of shrimp continue to survive & grow to fully harvestable size. Investigations conducted at ICAR-CIBA has revealed that there was no association of RMS with known shrimp viral infection.. Investigations have revealed no association with known shrimp viral infection as shrimp from RMS affected ponds tested negative for WSSV, IHHNV, IMNV, TSV, YHV, MBV, HPV and PvNV. Further, bacteriological examination of haemolymph samples of RMS affected shrimp indicated predominance of bacteria of *Vibrio* spp., such as *Vibrio parahaemolyticus* and *Vibrio azureus*. The population of anaerobic bacteria in the gut of RMS affected shrimp ranged from $72\text{--}252 \times 10^{14} \text{ mL}^{-1}$. The bacteria identified based on 16SrRNA gene analysis as *Enterococcus faecium*, *E. hirae*, and *Lactobacillus plantarum*. Bacterial diversity of RMS affected shrimp gut examined by Denaturing Gradient Gel Electrophoresis (DGGE) revealed a number of uncultured bacterial sequences. Histopathological examination of the hepatopancreas was largely normal. However, some samples showed karyomegaly and increased inter hepatopancreatic tubular space with haemolymph infiltration, muscle necrosis, loosened lymphoid organ (LO) tubule cells and constricted lumen. Bioassay experiments carried out by feeding RMS affected shrimp tissue to healthy 13-14 g shrimp did not elicit any disease in the experimental shrimp. All the experimental animals were healthy and active even after 44 hrs of feeding RMS affected shrimp tissue like that of control animals. RMS affected shrimp showed recovery and appeared healthy and active after 155 hrs of transferring to wet lab in water with optimal parameters. Co-habitation experiment with healthy shrimp and the infected animals also failed to induce RMS.

In view of no evidence for the involvement of an infectious aetiology to RMS, the role of better management practices, especially with regard to pond preparation protocols adopted indicated that critical environmental parameters viz., TAN, $\text{NO}_2\text{-N}$ and turbidity in water and organic carbon in soil were significantly high in ponds that were not dried adequately and correlated with incidence of diseases such as RMS and crop failure.

Size variation/ Growth retardation

More recently shrimp farmers have been reporting several cases of size variation/growth retardation in *L. Vannamei* grow out cultures. It is reported that viruses, viz., infectious hypodermal and hematopoietic necrosis virus (IHHNV), lymphoid organ vacuolization virus (LOVV), monodon baculovirus (MBV), hepatopancreatic parvovirus (HPV) and Laem-Singh virus (LSNV) are associated with slow growth and size variation in shrimp. In India, Madhavi et al. (2002) recorded multiple viral infections in shrimp with stunted growth. Rai et al. (2009) observed IHHNV, MBV and HPV associated with slow growth shrimp and stated that IHHNV could be one of the causes of slow growth in cultured *P. monodon*. In the event of white faeces syndrome affected animals there is a decrease in feed consumption and growth rates were reduced as revealed by average daily weight gain (ADG) for the whole crop operation of less than 0.1 g/day compared to 0.2 g/day in normal ponds. Feed conversion ratios (FCR) ranged from 1.7 to 2.5 when compared to 1.5 or less for normal ponds (Sriurairatana et al, 2014). Recently *Enterocytozoon hepatopenaei* found to be associated with size variation/growth retardation. Studies carried out at CIBA revealed that 36% and 42% of *L. vannamei* samples affected by stunted growth were positive for IHHNV and LSNV respectively. Histopathology investigation carried out on monodon slow growth (MSGs) affected shrimp showed pathognomonic lesions in different tissues, especially destruction of organ of Bellonci in eyestalk. Although the precise etiology of stunted growth and MSGS remains elusive, it appears that IHHNV and LSNV appear to play some role in growth retardation in farmed *L. vannamei*.

Summary

The Indian aquaculture production has substantially improved during the last ten years, especially after the introduction of the exotic Pacific white shrimp. However, the intensification of vannamei farming has exacerbated the epizootics and emergence of new diseases of known and unknown aetiologies, affecting productions and profitability. IMNV has recently been reported from many of the farms in India. Hepatopancreatic microsporidiosis due to a new microsporidian parasite “*Enterocytozoon hepatopenaei* (EHP)” associated with growth retardation resulting in reduced farm productivity has emerged in the Southeast Asian region since 2009. Disease syndromes of unknown aetiologies such as white faeces syndrome, running mortality syndrome, growth retardation have been significantly contributing to morbidity and productivity of *L. vannamei* grow-out cultures. Scientific literature has evidence that incidence of these aquaculture ecosystem related syndromes could be significantly minimized with biofloc shrimp farming technology.

HEALTH MANAGEMENT IN BIOFLOC SHRIMP CULTURE SYSTEM

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Shrimp aquaculture is a highly profitable export oriented industry that has undergone tremendous changes before evolving to its present form. Particularly culture of *Litopenaeus vannamei* has got wide acceptance in many parts of the world due to its several positive attributes including its suitability for high intensity culture practice. Biofloc based culture of shrimp is a recently developed practice where shrimps can be grown in high intensity without affecting the environment. Rather, the animals grow faster and grow healthy through improved immune and digestive system. Through low water exchange, pathogen entry from the external environment is prevented to large extent. While better growth is achieved from the already existing protein components of the floc component, the cell wall component of microorganisms act as immunostimulant to increase disease resistant. Biofloc has also been observed to have direct action on pathogens due to increase quorum sensing ability of beneficial bacteria and additionally due to their probiotic effect. Instances of low or no disease prevalence in biofloc system compared to the control system has been reported.

Disease is an undesired factor that always threatens the success of an aquaculture practice. Shrimp aquaculture throughout the world has witnessed several disease outbreaks and economical losses due to existing and emerging pathogens. Particularly, the high intensity culture practices are highly susceptible to different disease conditions and require special attention. Disease outbreak is merely not related to the presence of pathogens but also as a consequence to adverse environmental parameters. These unfavorable water parameters may weaken the immune system of the host for which even the opportunistic pathogens can take advantage and cause disease. Therefore, it is essential to keep the pathogens at bay and at the same time maintain good water quality to keep the immune system of aquatic animals active.

High intensity culture practices produces stress and thus reduces disease resistance capacity. Therefore, alternatives like a biofloc system should be there to compensate the stress by nullifying the effect of toxic agents like ammonia and nitrite. A biofloc system thus maintains good water quality and at the same time also directly acts on pathogens to keep them under control.

In spite of several added advantages, biofloc culture system is not free from disease risk. Rather, this high stocking density system requires special attention and care to keep it away getting infected by pathogens. Several precautions and special care are necessary to always keep the pathogens away from the culture system. At the same time, culture conditions should also be kept as optimum to prevent any kind of imbalance that will result in providing chance for opportunistic pathogens to become active.

Effective preparation of pond is the initial step

Pond preparation is an important step in shrimp aquaculture practice to eliminate pathogens from the system. There should be at least a month gap between two culture periods. Pond bottom soil which contains mostly unutilized feed and other wastes, should be removed. The bottom then should be left to dry enough. General processes like plowing, liming etc should effectively be carried out based on pond size and soil condition.

Use of SPF stocks are highly desirable

Prevention is always better than cure. Inclusion of pathogens in a biofloc system should completely be avoided. Therefore, larvae from specific pathogen free brooders should be used as a first step to avoid at least the known diseases. It will be possible to keep the culture environment free from pathogens through the adoption of stringent biosecurity. Therefore, use of SPF larvae will ensure a disease free condition for the harvest of a crop. At present it has been possible to obtain SPF *L. vannamei* and therefore, it should be the preferred species for culture in biofloc based culture system.

Selection of healthy larvae can avoid stress in pond system

In the beginning, healthy larvae should be selected for stocking in ponds. Healthy larvae in general will have better disease resistance capacity and these can better cope up with the adverse pond environmental conditions. Both salinity and formalin tolerance should be carried out to select healthy larvae.

PCR testing of larvae for presence of pathogens is a healthy practice

Though SPF stock ensures disease free status, it will be still better to check the larvae for their final status before stocking. This is more important when the brooders are reared in ponds in an illegal way. If the brooders are reared in ponds, these are no more considered as SPF and every possibility of getting infected by the existing pathogens of the environment. Moreover, SPF status is only against a limited number of pathogens. Therefore, in addition to the existing known pathogens, the larvae should also get tested for the presence of some emergent pathogens like AHPND and EHP.

Inclusion of a nursery biofloc based system is essential

Larvae are more susceptible to pathogens due to their under developed immune system. Particularly the initial 30-40 days of culture period is more important. Moreover, hatchery provides the utmost suitable environmental parameters which suddenly changes in a pond and the larvae undergo severe stress condition. Even some period during the beginning of biofloc development, the flocks are not matured enough to provide immediate immunity. Therefore, inclusion of a nursery phase will provide sufficient time period for the larvae to get acclimatized to the conditions and develop their immune system to withstand the adverse pond environmental conditions. During this period it will be still better to maintain the biofloc condition to make the larvae more healthier and quick development of their immune system. These larvae when released in ponds find no problem in getting quickly adjusted to the system and providing maximum possible survivability.

Maintenance of stringent biosecurity measures is a must

Though biofloc cultured animals are comparatively resistant, their under developed immune system (non-specific) is not suitable to challenge the highly virulent pathogens (such as WSSV). Stringent biosecurity measures are therefore should be put in place to avoid any entry of pathogens from the external sources. The initial water used for culture practice should be filtered and treated sufficiently to ensure killing of all existing pathogens. The water then should get matured by addition of beneficial microflora. Once the biofloc matures, then it will be possible to maintain zero water exchange condition. Till that period sufficient care should be taken for the treatment of the water. Ponds should be fenced properly to avoid the entry of unwanted animals those are possible sources for disease spread. All the man, machine and material should be sufficiently sanitized while moving from one pond to the other during sampling.

Efficient feed management can avoid most of the problems

Feed management is an important aspect in shrimp aquaculture practice. Particularly the biofloc based system provides natural feed and in this feed management is more desirable. A majority of the pelleted feed remain underutilized and these can provide nutrients to some of the pathogens like acute hepatopancreatic necrosis disease (AHPND) *Vibrio parahaemolyticus*. This also becomes a nutrient for many of the other bacteria, fungi and parasites. Regular check tray observation and feed adjustment through periodical biomass determination by sampling can provide clues for accurate feed use.

Indiscriminate use of chemicals/materials should be avoided

Biofloc based culture system is more considered as a self-sufficient system where augmentation of most of the shrimp requirements through natural micro biota takes place. It is unnecessary to add any other materials, chemicals from the external source. The beneficial microorganisms produced through the development of biofloc can act both as probiotics and bioremediators. The indiscriminate use of chemicals may produce undesirable environment and reduce the number/kill the beneficial organisms. Therefore, these are undesirable in biofloc-based system.

Use of antibiotics in biofloc based system should strictly be avoided

Usually the biofloc system will produce many heterotrophic bacteria and maintain good diversity. It has been seen that in this way it does not provide any space for the pathogens to grow. Even if bacterial problem is suspected, antibiotics should never be used in a biofloc based system. Along with pathogens, antibiotics will also kill the beneficial heterotrophic bacteria. Moreover, improper use of antibiotics can create drug resistance strains, which will have a negative impact on export of material.

A central drainage system will be highly beneficial

Though biofloc system enables waste utilization, natural decay of floc and other materials becomes undesirable. These material can support the growth of pathogenic microorganisms particularly bacteria, fungi and parasites. Therefore, the pond should have a

central drainage system to pump out settled materials from the bottom. Periodically these waste materials should be taken away through an efficient central drainage system.

Regular disease monitoring programme should be in place

It is a healthy practice to periodically monitor the culture system for the presence of pathogens. Random samples of both water and animals should be collected for testing. While the animals should be tested for all possible known pathogens, the water sample should be tested for pathogenic bacteria. Animals should also be regularly observed in the check tray. Ponds should be routinely observed for any abnormal behaviour of animals. If any kind of disease is suspected, immediate care should be taken to prevent it or confine and avoid the spread.

Pathogens/Practices	Sample type	Period	Test	Desired result
OIE listed pathogens: WSSV, IHNV, IMNV, YHV, TSV, NHPB, AHPND	Live/moribund animals Or Pleopods/faecal strings	Bi-weekly	Nested PCR/ Real time PCR	Confirmed negative
Non-OIE listed pathogens: EHP, DIV1	Live/moribund animals Or Faecal strings	Bi-weekly	Nested PCR/ Real time PCR	Confirmed negative
Bacterial count	Pond water and/or haemolymph	Bi-weekly	Total Plate count Selective plate count (Vibrios)	Good diversity (in water) and absence of pathogenic bacteria
Parasites/Fouling organisms	Scrapes from body Water sample	Bi-weekly	Microscopy	Absence of parasites/Fouling organisms on body Less number of ciliates in water
General observation	No sampling – direct observation of animals in water /Check tray	Any time	Visual	Very active, no external symptoms, intact organs, no deformity

Conclusion

Biofloc based culture system has several advantages, particularly due to high yield. As the demand for food and cheap protein source has constantly been increasing, this high density culture system looks very ideal to meet the demand. Particularly, its ability to maintain good water quality and stimulate immune system can make the culture environment comparatively disease free. However, this high intensity system also provides challenges specifically from disease point of view. Pathogens like WSSV, AHPND and EHP can be highly dangerous and bring severe mortality or growth retardation. Specific health management practices should therefore be adopted to avoid unpleasant situation.

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EFFECT OF BIO-FLOCS ON THE SHRIMP IMMUNE SYSTEM

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The bioflocs are consortium of particulate matter formed predominantly by a biota of aerobic and heterotrophic bacteria, protozoa, microalgae, metazoan, exoskeletons, faeces and remains of dead organisms. The diverse microbial community present in the biofloc system acts like natural probiotics and stimulates the non specific immune activity. Immunological potential level of biofloc reared animals were reported that the microbial cell components and their metabolites can act as immunostimulant that enhances the shrimp innate immune system and provide improved protection against pathogens. It has been already established that, bacteria and some microalgae produces exopolysaccharides (EPS) such as uronic or pyruvic acid under stressed condition, and these polymers are responsible for the adhesion and aggregation of dispersed cells of bacteria and microalgae, called bioflocs.

Biofloc and periphyton-based complex microbial systems in shrimp and fish aquaculture system without water exchange are found to be effective to maintain low levels of ammonia and nitrite as these systems harbor a variety of beneficial probiotic and nitrifying microbial communities. These microbial communities plays a major role which maintains stability and control of microbial populations, maintain stable water quality parameters, eliminate the presence of stressors like $\text{NH}_3\text{-N}$, $\text{NO}_2\text{-N}$, $\text{NO}_3\text{-N}$, through degradation of waste organic matter .etc., not only these provide a suitable environment and minimize water exchange and decrease the risk of disease occurrence, but also improves the immunity resulting in high health, growth and survival of animals.

Basics of shrimp immunity

As shrimps lack adaptive immunity, they depend largely on the innate immunity, which comprises of humoral and cellular immune activity. The innate immune system activity is based on the pattern associated molecular patterns (PAMPs) and pathogen recognition receptor (PRPs). The humoral immune system recognize the pathogen by prophenoloxidase cascade activation, clotting factors activation, and by the activation of antimicrobial peptide, the cellular immunity includes the activation of phagocytosis, nodule formation and encapsulation. Non-specific defense mechanism of shrimps response to pathogen intrusion via pattern recognition proteins (PRP) recognizes the conserved polysaccharides present in different probionts namely, β -1, 3-glucan (β G) from yeast, lipopolysaccharide (LPS) from Gram- negative bacteria, and peptidoglycan (PG) from Gram-positive bacteria and in turn trigger cellular and humoral responses. The major

proteins that function as PRPs in crustaceans are

- (1) β -glucan binding or recognition protein (β GBP, β GRP)
- (2) LPS and glucan binding protein (LGBP)
- (3) Gram-negative binding or recognition proteins (GNBPs or GNRPs)
- (4) Peptidoglycan recognition protein or peptidoglycan-binding protein (PGRP or PGBP)

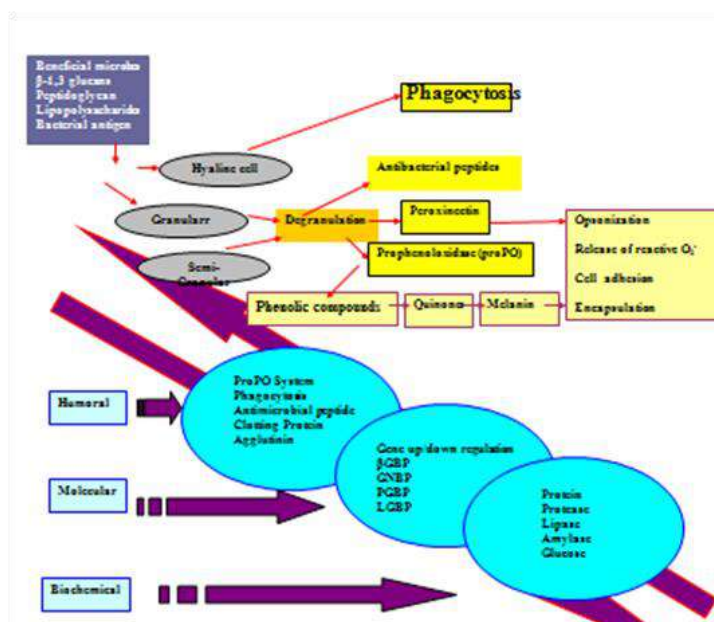


Fig. Immune mechanism involved during bacterial derived immunomodulations

It is known that the PRPs, like β GBP, LGBP and PGRP which recognize and respond to microbial intruders, are involved in activating the innate defense mechanism especially the humoral defense cascades in shrimp. These responses are nonspecific and target a broad range of bacterial and fungal invaders. Crustacean haemocytes play important roles in the host immune response including recognition, phagocytosis, melanization, cytotoxicity and cell to cell communication. Classification of the haemocyte types in decapod crustaceans is based mainly on the presence of cytoplasmic granules in the hyaline cells, semigranular cells, and granular cells. Each cell type is active in defence reactions, for example, the hyaline cells are chiefly involved in phagocytosis, and the semigranular cells are the cells active in encapsulation, while the granular cells participate in storage and release of the prophenoloxidase (ProPO) system and cytotoxicity. The haematopoietic tissue has been described in several crustacean decapod species and shown to be the haemocyte-producing organ.

Shrimp immune modulation in biofloc system

Biofloc technology can be seen more as a mechanism, which provides shrimps a chance to keep the immune system active at all times, as they get exposed to various microbes. Shrimps gut is exposed to natural micro flora, which provides nutritional, immunological benefits, especially on preventing the infection from the pathogen by competitive exclusion, neutralization of toxins and bactericidal activity. The natural probiotic effect in biofloc provides antigen to trigger the immune response in gut. Many reports suggest that wide range of beneficial microbes or their cell wall components and metabolites is improving the innate immunity and can be employed in the health management of fish and shellfishes. Biofloc has been proved that it is rich in natural source for bioactive compound and microbes thus it is proved to be an efficient immune inducer in the shrimp. Biofloc increases the survival rate even when there is an infection.

Disease resistance

There is diversity of microbes present in the system which is both beneficial as well as pathogenic to the cultured species and quorum sensing of the microbes that significantly elevates the number in the system. This antagonism activity between the pathogen and other heterotrophic bacteria limits the pathogen to multiply. *Vibrio* is an opportunistic bacterium, they may grow at higher rate during initial culture period but as the biofloc water matures the system builds a strong diverse environment against the pathogenic bacteria.

In our study a trial has been made to check the immunity of biofloc reared shrimps by challenging them with the known pathogen *V. parahemolyticus*. The bacterial suspension for challenge has been prepared as per the protocol and when ABW of 15-18g biofloc reared shrimps were injected intramuscularly and evaluated against normal saline injected shrimps as control. The cumulative mortality was found to be significantly lower in the biofloc reared animals.

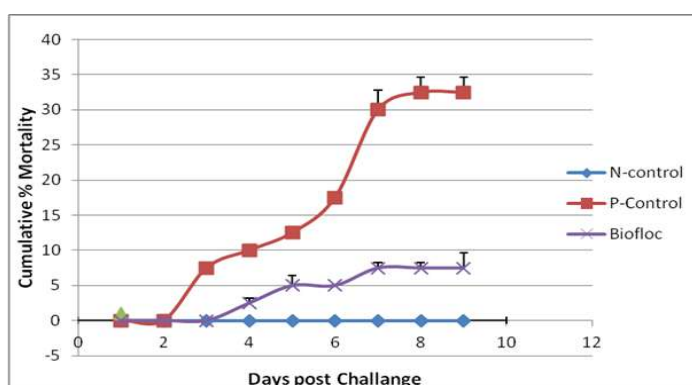


Fig 2. Cumulative mortality of *P. vannamei* shrimps challenged against *V. parahemolyticus*.

Immune Gene Expression

Transcripts of target immune genes were measured by qPCR. Q-PCR results revealed appreciably enhanced mRNA expression of certain genes when *P. vannamei* were cultured in a biofloc system. Superoxide dismutase (SOD) is an enzyme which alternatively catalyzes superoxide anions (O_2^-) to molecular oxygen (O_2) and hydrogen peroxide (H_2O_2). It has diverse pharmacological activities and also shown to play a crucial role in antioxidant protection. Prophenoloxidase activation is a major part of immune system in shrimp. The upregulation of this gene indicates that the bacteria associated biofloc play the major role in enhanced immune activity in the shrimps (Up regulation of immune genes in biofloc reared shrimps- hemocyanin-5.9 fold, lysozyme 3.3 fold, - Serine proteinase-1.65 fold, Glutathione peroxidase,5.25 fold, SOD- 5.9 fold, Prophenoloxidase-1.29)

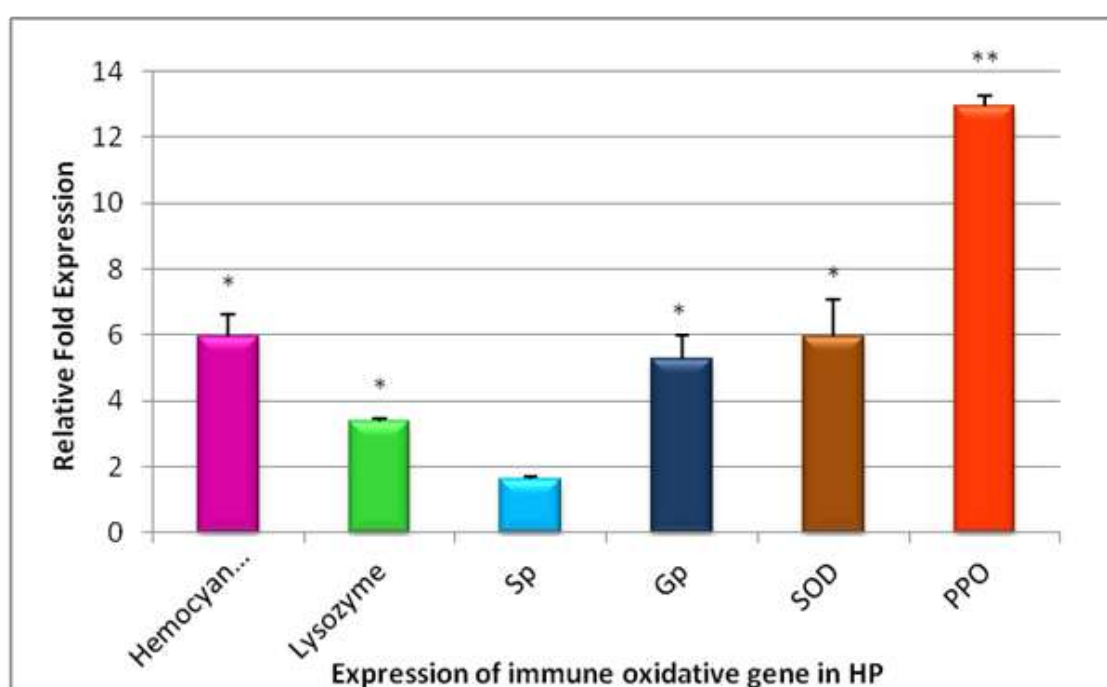


Fig 3. Comparative mRNA expression levels of immune gene hemocyanin, lysozyme, serine proteinase, Glutathione peroxidase, superoxide dismutase, prophenoloxidase in *P. vannamei* reared in biofloc systems. A relative expression value of 1.0 indicates no change in response to treatment.

A similar study revealed six genes that are involved in the innate immune response of shrimp [proPO1 (prophenoloxidase 1), proPO2 (prophenoloxidase 2), PPAE (prophenoloxidase activating enzyme), SP1 (serine protease), mas (masquerade-like serine proteinase), ran (ras-related nuclear) through mRNA expression (Su-Kyoung Kim and In Kwon Jang, 2015)

Following attributes of bio-floc technology helps in prevention of diseases

- Bio-floc technology (BFT) systems were developed to minimize effluent discharge,

protect the surrounding water resources and improve farm biosecurity. Almost zero or minimal water exchange is good for biosecurity and pathogen exclusion.

- Continuous aeration in the system mixes water thus avoiding stratifications and the stable water creates better stress-free environment.
- Diverse beneficial bacterial community in the bio-floc can stimulate the non-specific immunity and limit establishment of pathogenic strains
- The settled solid sand suspended solid waste in this system is recognized and is removed from the bio-floc system thus preventing any risk of disease from the sludge.
- Priming of immune system of the host helps in immunomodulation and disease resistance in the animals reared in this system

Gene expression measured in mysis, postlarvae and adult *P. vannamei* was found to be enhanced in the presence of bio-floc. Our studies suggest that microbes associated with bio-flocs may enhance expression of certain immune-related genes.

Bio-flocs as biocontrol measure

The “natural probiotic” effect of bio-floc could act internally and/or externally against, *Vibrio* sp. and ectoparasites. The regular addition of carbon in the water is known to select for polyhydroxy alkanoates (PHA) accumulating bacteria which produces biodegradable polymer storage products, like poly- β -hydroxybutyrate (PHB), having antibacterial or biocontrol properties that provide immunity to the host. Though bio-floc concept is yet to be practiced by many in India, probiotics is an established business at present in Indian aquaculture.

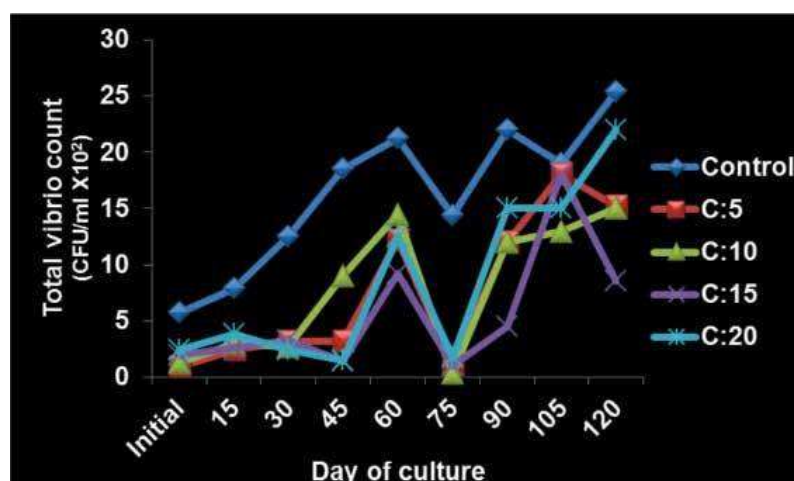


Fig 4. Reduction of *Vibrio* load in variuos treatments

Probiotic interventions of *L. rhamnosus* and *B. subtilis* were found to be advantageous in terms of better growth and survival rate and the expression of certain immune related genes in response to microbial interventions were significantly up-regulated explaining the

possible immunomodulations and in turn better protection in fish (Panigrahi *et al.*, 2007b; 2011). Beneficial communities in biofloc system control the pathogenic *Vibrio* population (Crab *et al.*, 2012).

Heterotrophs versus Pathogenic bacteria

There is diversity of microbes present in the system which is both beneficial as well as pathogenic to the cultured species and quorum sensing of the microbes that significantly elevates the value in the culture system. In Biofloc system, shrimps were constantly exposed to range of microbes, while most of them are harmless to the shrimps but few species were pathogenic. *Vibrio* is an opportunistic bacterium; which may grow at higher rate during initial culture period but as the biofloc water ages the system builds a strong diverse environment against the pathogenic bacteria. When the floc settle in the bottom and starts decomposes, it provides an opportunity for pathogenic bacteria to increase in the system which may also create an issue in shrimp's health. Many investigations had already showed that these beneficial bacteria can be effectively used against luminous Vibriosis.

Quorum sensing mechanism and its inhibition by Quorum quenching for control of pathogenic bacteria

It has been proved that bacteria also highly interactive creatures, which exhibit complex cooperative behavior like conjugal plasmid transfer, bio-film and virulence. Many of these behaviours are regulated by quorum sensing. Among bacteria, each one is capable of producing a signaling molecule (inducer) and each has a receptor too for the inducer. When the inducer binds to the receptor, it activates the transcription of certain genes including those responsible for the synthesis of the inducer itself. Once a threshold concentration is attained, activation of the receptor leads to a signal transduction cascade to switch on specific genes in the bacterial cells, leading to a coordinated population response. Through these QS, pathogens can increase their virulence and bring mortality.

Development of quorum sensing inhibitors may potentially be useful in inhibiting the growth or virulence mechanisms of bacteria in different environments during shrimp culture. Since appearance of antibiotic-resistant bacteria is universal, there is an increasing need for novel strategies to control infectious diseases like vibriosis. Bio-film bacteria (bacteria living in the bio-film mode of growth) tolerate conventional antimicrobial treatments. Biofloc system contains many of the naturally developing beneficial bacteria and algal components. All these organisms in combination and the enzymes those they release can act as inhibitor for pathogenic bacteria like *Vibrios* to bring higher survival.

Mechanisms of action of the beneficial bacteria

Beneficial bacteria in the biofoc system can act as Probiotics which then can act in different ways and various mechanisms as proposed for the mode of action of probiotics that

is given below.

- Antagonism (By affecting the balance between pathogenic and harmful microbes and beneficial microbes)
- Competition for adhesion sites and nutrients with other microbes in the niches, for chemicals and available energy
- Production of inhibitory compounds in host or in culture medium
- Probiotic bacteria can directly take up or decompose the organic matter or toxic material and improve the quality of water by increasing DO, preventing off flavour, reduction of nitrogen & phosphorous level, reducing blue green algae etc.
- Interaction with phytoplankton and improving natural productivity
- As a source of macro and micronutrients
- Enzymatic contribution to digestion - The microbial cultures produce a variety of enzymes like amylase, protease, lipase, xylanase, and cellulase in higher concentrations compared to the native bacteria
- Immunomodulation (Microbial products eliciting immune response at humoral, cellular and molecular level) results in improving the animal's over all health by possible exclusion of pathogens from the production system, enhancement animal's physical condition, appearance, average size and weight of the animal and restoration of reduced appetite and feed consumption. However, modes of action are sometimes circumstantial and based on empirical arguments.

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APPLICATIONS OF BIOFLOC TECHNOLOGY

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As we trace the progress of aquaculture through the last 30 years we see that the aquafarming is slowly but surely evolving from flow through, to water exchange, to zero exchange. This trend towards zero exchange has been brought about by various factors, majorly, degradation of water quality in natural waters due to increasing industrial and agricultural activities as well as rapid urbanisation. Intensification of aquaculture gives rise to various pathogenic blooms in the culture water, which when released without treatment, further contributes to biological pollution of the natural waters. Hence water which was seen as a free resource has now become an expensive commodity which needs to be treated and sterilized before and after use.

Why do we need to change water?

When we feed the aquatic animals with feed which has some protein content, the animals and the feed wastes release ammonia in the water, this ammonia is toxic to the animals and can be lethal if not controlled. There are various naturally occurring organisms which feed on ammonia and utilise it for their growth and energy, amongst the most easily visible is algae which convert ammonia into algal biomass by using the energy from the sunlight. For a long time aquaculture systems have been run by controlling these algal blooms with limited water exchange. Algal blooms being sunlight dependant go through a day and night cycle causing variable oxygen demand, fluctuating pH etc. within every 24 hour cycle causing stress to the animals if not controlled. The nitrogen conversion capacity of these algal systems is also limited to the surface area of the pond.

Zero Exchange Technologies

With the rising costs of both water and land there is a lot of pressure to produce more fish/shrimp per unit of water and land. Bacteria based systems were found to be more efficient as they are not limited to sunlight. There are two major bacteria based technologies available today, one is Recirculating aquaculture system, which uses Autotrophic bacteria and the more recent is Biofloc technology which uses Heterotrophic bacteria.

RAS requires a high capital expenditure because of external biofilters and other water purifying equipment such as settlers, foam fractionators, Ultra violet, Ozone etc. These systems are being used for more than 3 decades and most functions can now be automated. Biofloc is a new technology which requires much less capital expenditure but higher and better trained management input. Other advantages of this technology are lower feed requirement and better immunity. Due to these advantages, this technology is becoming very popular and has many applications.

Hatchery application

In 2007 our trials with Scampi Zoea have shown more than 50% reduction of Artemia consumption at double density of Zoea. Recently *P.vannamei* PL grown in Biofloc have shown less size variation and have been tested proved to have better immunity as per challenge tests conducted by CIBA.

Nursery application

Zero exchange high density nursery reared juveniles have been seen to have minimum size variation and perform better due to better developed immune response systems. This is a very good biosecurity tool for the farmers and a perfect transition phase between the Hatchery and Farm. Nurseries should be located away from the farms to avoid exchanging pathogens from overlapping crops.

Farm application

This is an excellent technology for getting a maximum production per cubic meter of water and square meter of land, depending on the sludge removal and aeration capacity of the system 2 to 6 kg of shrimp or 10 to 50kg of fish can be grown in these farming systems. Continuous monitoring of DO and Floc levels is critical in these systems.

Brood stock maintenance

Brood stock maintained in zero exchange Biofloc systems have shown to have better fecundity and fertilisation. Large numbers of animals can be maintained with minimum requirement of water and space. The cost of Biosecurity for these systems is much lower than other available systems. Artemia biomass grown on Biofloc is an excellent feed for broodstock.

As a feed supplement

Biofloc biomass is a high protein live feed which can be produced separately and fed to animals in high density culture systems with very good results. Biofloc biomass can also be incorporated into feed pellets or moist feeds for better growth and health of animals and reducing the requirement of fish meal component in feed formulations.

Primary feed production

In the new Aqua-mimicry type of systems which promotes the growth of rotifers and copepods, Biofloc in the form of heterotrophic blooms formed by addition of fermented rice bran is the primary source of feed for these animals.

Effluent treatment

Biofloc technology can be used to treat waste water with very high ammonia load. This waste ammonia can be converted to fertilizer or fish feed supplements by addition of carbon and oxygen.

Overall, this new technology has enormous importance in many different applications. With increasing efficiency in the use of Biofloc we will have more eco-friendly and less wasteful systems to address the future of food security.



ECOSYSTEM BASED BIOFLOC SHRIMP FARMING: NURSERY-REARING EXPERIENCES

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Shrimp culture remains the economic engine of the aquaculture sector in India, and the industry earned export worth USD 4.85 billion with an all-time high of 0.69 million tons in the year 2018 (FAO, 2019). Pacific white shrimp, *Penaeus vannamei*, is the major contributor (90%) in the Indian shrimp export basket. The specific pathogen-free (SPF) *P. vannamei* has revolutionized the Indian shrimp sector since its introduction in the year 2009 and resulted in a spectacular growth rate (13%) over the past two decades. ICAR-Central Institute of Brackishwater Aquaculture, Chennai has been conducting Frontline Demonstrations on Biofloc based Nursery rearing Technology for Pacific white shrimp *Penaeus vannamei* in different parts of the country with support from the Department of Biotechnology, Govt. of India. This innovative eco-based technology is a sustainable system in which the waste materials are recycled into microbial flocs. This unique feature translates into better water quality and pond environment. The microbial flocs thus formed are a source of protein for the shrimps, thus reducing the feed cost.

The BFT based production system can be divided into nursery and grow-out farming system. Each stage has its own important role for the success in production. In order to achieve higher survival and reduction in the grow-out period, an intermediate nursery phase is essential. Nursery phase is defined as an intermediate step between hatchery-reared early post larvae and grow-out phase and it has several advantages such as optimization of farmland, increase in survival and enhanced growth performance in grow-out ponds. BFT has been applied successfully in nursery phase and grow out phases in different shrimp species such as *P. vannamei*, *P. monodon*, *F. paulensis*, *F. brasiliensis* and *F. setiferus*. CIBA has developed BFT and demonstrated the technology in the field with SOP (Standard Operating Procedure).

Nursery rearing in shrimp farming is an intermediate phase between the hatchery and grow-out phase and introduced in the 1970s (Parker et al., 1974; Samocha and Lawrence, 1992). However, most of the shrimp farmers avoided this phase as it believed to increase the cost of production, and enhance the handling stress to the rearing stock. Later, when recurrent crop losses increasingly constrained the shrimp industry due to the emerging diseases, the nursery phase regained momentum (Samocha, 2010) as a novel strategy for better productivity and profitability. These seed bank systems have multiple advantages such as it provide accurate stocking inventory, uniformity of size at harvest, compensatory growth during grow-out stages with short culture duration and higher grow out yields, better control over biosecurity (Arnold et al., 2006; Ballester et al., 2007; Correia et al., 2014; Wasielesky et al., 2013). Adoption of shrimp nursery rearing enhances 20-30% increases in shrimp farm productivity compared to the traditional single-phase grow-out system (Garcia Abad, 2015).

Approaches of nursery rearing

With the adoption of nursery rearing, various management practices such as zero water exchange nursery (Emerenciano et al., 2012), clear water nursery systems (Esparza-Leal et al., 2015), biofloc based nursery systems (Panigrahi et al., 2020), hybrid nursery systems (Tierney and Ray, 2018), etc. are evolved. The nursery performance of commercially important penaeid shrimp such as *P. vannamei* (Panigrahi et al., 2020), black tiger shrimp, *P. monodon* (Arnold et al., 2009), pink shrimp, *P. paulensis* (Ballester et al., 2007), *P. brasiliensis* (Emerenciano et al., 2012; De Souza et al., 2014) is well documented

There are two kinds of approaches for nursery rearing of penaeid shrimps. The first approach is conventional based nursery rearing in which early post larvae is reared with pond or tank with stocking density of 300 to 3000 nos/Cu. M. On the other hand, high tech nursery system with biofloc (BFT) and recirculatory (RAS) system with stocking density of 3000 to 20000 nos/Cu. M. can be achieved with technical control and expertise.

Advantages in nursery phase

- The compensatory growth gain recorded in grow out farm subsequent to nursery rearing will help to reduce crop culture duration.
- Uniformity of size at harvest and higher yield.
- Adoption of shrimp nursery rearing enhances 20-30% increases in shrimp farm productivity compared to the traditional single-phase grow-out system
- The primary advantage observed is related to a better nutrition by continuous consumption of biofloc, which might positively influence grow-out performance as compensatory growth phenomenon.
- Optimization of farm facilities provided by the high stocking densities in BFT nursery phase seems to be an important advantage to achieve profitability in small farms mainly in cold regions or when farmers are operating indoor facilities.
- Study revealed that presence of bioflocs resulted in increase of 50% in weight and almost 80% in final biomass in Pink Shrimp, *F. paulensis* early post larval stage when compared to conventional clear-water system.
- Another study on *F. brasiliensis* post larvae reared with or without pelletized feed in biofloc conditions during 30day of nursery phase, recorded 40% higher growth performance and survival compared to conventional clear-water continuous exchange system.
- Immune systems of shrimps are improved in the biofloc system due to the bacterial community, thus presenting a higher health status to the animals.
- It improves productivity, natural food, FCR, economic gain and reduced costs (15-20% lower cost of production).
- Heterotrophic bacteria reduce toxic metabolites (NH₃-N, NO₂-N) in the nursery-rearing phase.

Standard operating procedure for nursery rearing

SOP for nursery rearing includes nursery design and construction, bio-security, species selection, stocking density, management practices, feed management, carbon addition strategy, water quality management and sludge removal.

(I). Nursery design and construction

Pond can be either with concrete or HDPE lining work. Facilities for higher uninterrupted aeration should be in the farm for aeration needs in the biofloc tanks. For this purpose, high pressure fine diffuser or paddle wheel aerator can be used. In a biofloc system, frequent settling of sludge will be there and periodic removal of it is necessary to maintain quality pond environment. This point should be taken into account while constructing the pond. A central drain must be provided for the periodic removal of the accumulated sludge.

(II). Biosecurity measures

Biosecurity measures should be in place to avoid any entry of pathogens from the external sources on horizontal route. Proper fencing (Crab, bird fencing) sanitized equipments and/or machineries and regularized personnel movement.

(III). Species selection and stocking density

Suitable species, which is appropriate for rearing, should be selected. Disease free certified seeds (specific pathogen free) must be selected for rearing in the nursery. Post larvae of size 5-8 can be selected for rearing in the nursery system. Stocking density of 10000/m² can be stocked into the system for rearing.

(IV). Biofloc generation and maintenance

To generate the biofloc in the nursery rearing system as a first step the culture water is chlorinated with bleaching powder, @20-30ppm active chlorine. Further fertilization is done with suitable materials at 5-15ppm dose. Addition of biofloc consortium at a dose of 0.1-0.3gm/Cu.M; along with addition of carbohydrate sources (Mollasses, rice bran, atta etc.) at a dose of 2-5ppm on daily basis.

(V). Feed management

The size and quantity of feed should be regularized from period to period during nursery phase with addition of carbon source during the nursery phase. This calculation can be done depending upon the accumulation of the TAN levels in the nursery system.

(VI). Water quality management (Zero/Minimal water exchange)

Regular monitoring of water quality is essential to rectify/adjust the accumulation of nitrogen metabolites, floc level and other inputs in the nursery system.

(VII). Sludge management

Excessive and degraded floc increases the production of toxic gases like H₂S, which is highly

toxic to aquatic organisms, higher vibrio load at the pond bottom. Therefore, removal of sludge from the pond bottom should be done in a routine basis.

Table 1: Nursery rearing performance with the biofloc inoculum supplied by ICAR-CIBA

	TRIAL 1	TRIAL 2
Species	<i>Penaeus vannamei</i>	<i>Penaeus vannamei</i>
Larval stage	PL 7	PL 7
Stocking density	3000 PL/m ³	3500 PL/m ³
Growth	0.92 ± 0.15 g	0.91 ± 0.15 g
Survival	94.20 ± 2.86 %	92 ± 2.3%
DOC	28-30 Days	28-32 Days



Fig 1: a) Biofloc nursery rearing tank, b) Floc density estimation in an Imhoff cone,
c) Nursery reared seed harvested.

Table 2. Growth performance of shrimps in Biofloc based nursery-rearing system

Production performance	Control	Biofloc based nursery
Stocking (Nos/m²)	5000 to 10000	5000 to 10000
Floc volume	0-2 ml	3-12 ml
Nursery rearing days	3-4 weeks	3-4 weeks
Survival (%)	55-80 %	78-92 %
Average body weight at harvest	220-350 mg	320-400 mg
FCR	0.95--1.15	0.65—0.89

Source, GAA, 2009

Conclusion

Nursery rearing of post-larvae before stocking in grow-out ponds is becoming an evolving technology due to multiple advantages such as better control over biosecurity and higher yield during the grow-out phase. It is highly important to device location-specific nursery rearing technology based on the type of cultured shrimps or fishes, its culture attribute and feeding preferences.

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INSIGHTS INTO THE BIOFLOC BASED SHRIMP GROW OUT TECHNOLOGY

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Biofloc based farming of shrimp is gaining momentum today. Although the principles of this technology was previously applied to the growout of fishes like Tilapia, the present scenario shows that it is suitable for shrimp farming also. This technology is being tested world wide for the sustainable production of shrimp and is providing promising results. In a biofloc system the waste materials in the pond (nitrogenous wastes) are converted into bacterial flocs which is high in protein. By doing so the toxic gases like NH_3 , NO_2 are removed from the system, which are otherwise detrimental to the cultured organism. The bacterial flocs also forms as a feed material for the species on culture, thus reducing the feed cost. Due to these advantages the biofloc based farming systems are considered as sustainable and ecosystem based technology.

The major advantages of the biofloc system are reduced feed cost, maintenance of water quality due to the action of the bacterial community, minimal water exchange etc. Apart from this, recent studies shows that the shrimps reared in the biofloc system possess a better immune response. The animals reared in the biofloc system have higher resistance to diseases and stress. Even though there are lot of advantages for this system, the adoption level of this technology for commercial rearing system is still in the infant stage. This may be due to the technological complexity and lack of knowledge about the system. However, in the present scenario, this technology is been tested in various levels at different locations and the results shows that it is possible to take a successful crop provided the necessary requirements are met.

Shrimp growout in biofloc technology: Overview

- In biofloc culture conditions, the major advantage is the reduced feed usage. In recent studies, It was estimated that more than 29% of the daily food intake of *L. vannamei* consisted of microbial flocs, decreasing FCR and reducing costs in feed.
- A 120 days trial of *L.vannamei* with different stocking densities, 150, 300 and 450 shrimp /m² had a survival of 92, 81 and 75%, respectively. Moreover, study performed in a heterotrophic-based condition detected no significant difference in FCR when feeding *L. vannamei* with 30 and 45% CP diets and 39% and 43% CP diets, respectively in with and without floc system.
- It is noticed that glucose or a combination of glycerol plus *Bacillus* as a carbon source in bioreactors led to higher biofloc protein content, higher n- 6 fatty acids. It is also documented that no significant differences in growth performance of shrimps fed with 44% CP under BFT and clear-water conditions.
- One of our studies conducted in ICAR-CIBA reveals the utility of reducing the protein in feed while customizing feed for BFT.
- In one of the trial in Malaysia, where different stocking densities of 40,60,80,100 and

130 nos/m² was tried in a bio secured biofloc module of HDPE lined ponds and yielded a production level of 9.75 to 15.43 tonnes/ha/crop with FCR of 1.32 to 1.74 (Taw and Saleh, GAA 2013). Energy input was found to be 355kg/hp to 643kg/hp.

- Biofloc based recirculatory aquaculture systems conducted at Ocean Institute at Hawaii by Moss et al., (2006) recorded a productivity of 7.5 kg/m³ with an average body weight 24.7 g in a stocking density of 300 nos/m³.
- When penaeid shrimps reared at 500 nos/m² recorded a survival of 78-81% survival with a production performance at 8.86-9.2 kg/m³(Samocha et al., 2007).
- Recirculatory Aquaculture System conducted at Texas A & M University by Samocha (2009) recorded a final productivity of 9.37 kg/ m³ with an average body weight 22.36 g from a stocking density at 450/m³.
- ICAR-CIBA static grow out system recorded a productivity of 5 kg/ m³ with an ABW 36-40g from a stocking density of 200/m³.
- Growth performance of shrimps reared at biofloc based Hitide farms in TamilNadu recorded 39 g average body weight with 19.78 t/ha/crop compared to 15.7 t/ha/crop with 31.68 g ABW at 60 days of culture.

Studies on the water quality parameters in the biofloc rearing system shows that the critical water quality parameters were in the optimum level. The total suspended solids were in the higher level due to the presence of the bacterial flocs. The major parameters like the ammonia nitrogen and the nitrite nitrogen were in the acceptable limits.

Table: Water quality parameters of intensive race way based Biofloc shrimp farming (Samocha et al., 2013)

Water quality parameters	Mean (± S.D.)
Five-days biological oxygen demand (mg/L)	48.0 ± 16.9
Alkalinity (mg/L)	170.8 ± 17.7
Total suspended solids (mg/L)	292.4 ± 75.5
Settleable solids (mol/L)	12.2 ± 3.5
Volatile suspended solids (mg/L)	193.4 ± 38.6
Turbidity (NTU)	116.2 ± 42.1
Total ammonia nitrogen (mg/L)	0.30 ± 0.14
Nitrite nitrogen (mg/L)	0.36 ± 0.29
Nitrate nitrogen (mg/L)	172.4 ± 91.7

Table: Growth parameters of intensive race way based Biofloc shrimp farming (Samocha et al., 2013)

Growth parameters	Range
Stocking (shrimp/m ³)	500
Stocking Weight (g)	3.6
Harvest Weight (g)	22.76
Weekly Growth (g)	2.13
Survival (%)	78-81
Yield (kg/m ³)	8.86-9.2
FCR	1.5
Water Use (L/kg)	139-150

Studies on the growth parameters showed that that any given stocking density the final yield was higher in the biofloc based rearing system than in the conventional shrimp farming system. The mean bodyweight and the final harvest tonnage was also higher in the biofloc ponds. The higher tonnage can be related to the higher stocking density in the biofloc system due to better water quality in these systems. Feed conversion ratio was much lower in the biofloc based system suggesting the intake of bacterial flocs and reduced feed usage. The survival rate did not differ much between the conventional and biofloc

Table: Production Performance of Biofloc based HDPE lined shrimp farm in Arc Biru Farm, Malasiya

Parameters	Biofloc 0.4 ha HDP	Semi Biofloc 0.8 ha HDPE	Conven 0.8 ha HDPE
No.of Ponds	2	19	19
PWA Energy (Hp)	14	24	20
Stocking density	130	110	83
DOC (days)	90	101	111
SR%	89.16	81.35	83.19
MBW (gr)	18.78	18.31	17.80
FCR	1.39	1.58	1.77
ADG(gr/day)	0.21	0.18	0.16
Avg Harvest tonnage	9006	12950	9616
Production (kg/ha)	22514	16188	12019

Table: Growth performance of shrimps reared in Grow- out system, Indonesia

Description	Stock Density (Nos./m ²)			
	660	130	200	130
Initial MBW (g)	4.9	1.7	0.18	0.18
Harvest Biomass (kg)	374.0	151.0	183	187
Final MBW (g)	13.8	18.4	14.3	14.3
Survival rate (%)	66	88	89	100
Productivity (kg/Ha)	61,883	21,001	25,432	18,993

Table: Comparative growth performance of Biofloc grow out trails in recirculatory and static system

Description	Ocean Institute Hawaii Moss (2006)	Texas A & M University Samocha (2009)	ICAR-CIBA Static system
Stocking Density	300/m ³	450/m ³	200/m ³
FCR	1.49	1.52	1.52
Size	24.7	22.36 g	36 -40g
Production	7.5 kg/m ³	9.37 kg/ m ³	5 kg/ m ³
System	RAS	RAS	Static

In the present day scenario, intensive studies are going on the optimization of the biofloc system and different models of the biofloc system are designed to maximize the yield and profit. In CIBA we have used the static system and the production performance were on par with the RAS growout systems in terms of average body weight and feed conversion ratio.

Table: Economics of Biofloc and autotrophic based system

	Biofloc	Autotrophic	Remarks
Production (MT)	22 MT/ha	21 M/ha	Increase in production = More profit
Growth (gms/day)	0.16 to 2.1	0.13 to 0.16	Large shrimp size
FCR	1.1 to 1.3	1.5 to 1.7	Lower FCR = lesser feed cost. FCR 0.1 = 4% of feed cost
Water exchange	Zero water exchange	Minimum or flow through	Energy saving in water pumping
Gross profit	> 35%	< 30%	The more the profit the better

Production cost	< 15-20 % than Autotrophic	Standard autotrophic	Less production cost= More profit
Feed mill – production	Less sale but more sustainable sale	Normal sale	Feed mill should include grain pellet for Biofloc with which sustainable sales could be secured

Table 8. Growth performance of shrimps reared at Hitide farm in Tamil Nadu, India

Sl.No	Particulars	Control Pond	Biofloc Pond
1	Pond Area (ha)	0.5	0.12
2	Stocking Density (nos/m ²)	60	60
3	Duration of crop (days)	149	160
4	ABW at final Harvest (g)	31.68	39.00
5	Yield (t/ha/crop)	15.73	19.78
6	Survival (%)	85	95
7	FCR	1.6	1.4

A comparative study conducted by CIBA between a biofloc pond and a normal pond showed better results in the biofloc pond. The average bodyweight was higher in the biofloc pond than in the control pond. The survival rate was higher in the biofloc pond with better FCR, which was lower than the control pond. These results shows that there is a definite positive effect of the bacterial flocs on the growth of the shrimps.

Experiences from a progressive farmer after adopting biofloc technology

Mr. Nishanth Reddy is a progressive farmer who took his family business as his profession. They are into the aqua business for the past 33 years especially the shrimp farming. In his beginning years the most problems occurred in the farming side was because of diseases of the shrimp. In his terms, the major problems were caused due to the White spot virus, Running mortality syndrome and White faeces syndrome. The most concerning one was the White faeces issue, which used to affect at the size of 3-10gm where the shrimp used to eventually die by converting into loose shell and White spot syndrome virus, which use to occur at any point of farming cycle. The losses were abnormal when it was affected the less than 7 grams which was before 50 days of culture. The first 50 days of culture was always

under threat and occurrence of disease was highly unpredictable inspite of good biosecurity as it is an airborne virus. At this point he joined ICAR-CIBA to learn more about the new avenues in the shrimp farming sector and especially the biofloc faming technology. After gaining the base knowledge from the institute, he started improvising the technique in his own level and farm conditions.

It was at this point he started to think about a second phase of hatchery, which was inspired from a plant nursery where he could grow the shrimp to a bigger size in a secured environment. By doing so the growth was faster and the concept of nursery was evolved from it. In his unique process of the nursery rearing technology the shrimps were stunted and not allowed it to grow much for the first 20-25 days as the beginning of the culture phase by stocking the PL brought from the hatchery. Later on the shrimp juveniles grown in the nursery system was later stocked in to the ponds as the conventional shrimp farming system. This method help him achieve compensatory growth of 10 grams in the first 30 days where he could reach breakeven in a very short time and reduce the risk of loss.

However, the problems associated with the white feces and running mortality still could not be managed inspite of stocking nursery seed. A root cause analyses revealed that the decomposed sludge in the pond bottom during the crop caused the maximum problems. At this point he started trials on a small sized HDPE lined ponds by stocking the same density of extensive farming being 30 pcs/m². He successfully harvested the shrimp without any problems and started second trial in 60 pcs/m², the result was repeated without any issues.

In the course of this farming method, he eventually started applying the Biofloc technology for the growout farming of the shrimp. The success in HDPE lined ponds encouraged him to stock higher densities and he gradually increased the densities from 60 pcs/m² to 200 pcs/m² by application of the biofloc method of farming. He successfully started getting better yields and have reached a maximum of 40 tons/hectare/crop. The disinfection of Lined ponds took only 1 week which led to 4 crops an year and have achieved a maximum density of 160 tons/hectare/year.



A glimpse of Mr. Nishanth Reddys farm

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CONTROLLING AQUATIC PATHOGENS THROUGH BFT: W.R.T LUMINESCENCE CAUSING *VIBRIO HARVEYI*

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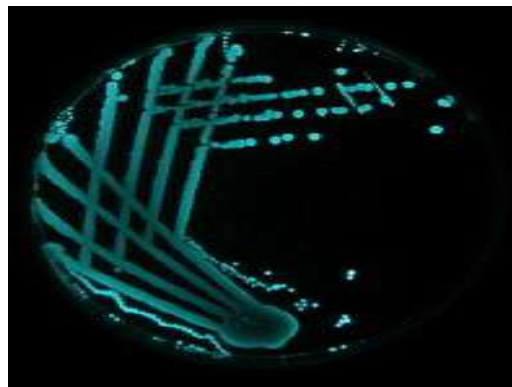
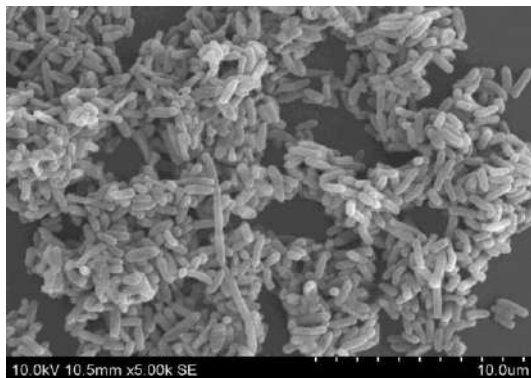
Introduction

Aquaculture is a quickly growing protein rich food-producing sector, but its growth is not without problems. During shrimp grow-out practices, considerable amounts of waste water effluent, containing uneaten feed and faeces that release ammonia and nitrite, which is toxic for both fish and shellfish (Crab et al. 2007). Conventional techniques used to overcome this problem are introduction of photoautotrophic algae into the grow-out system and frequent water exchange etc (Crab et al. 2007). These techniques, however, are lacking economical, technical, sustainable and practical competence. A technique that overcomes these shortcomings is the bioflocs technology. By adding carbohydrates into the shrimp culture system to increase the Carbon/Nitrogen ratio (micro-organisms tend to form amorphous aggregates called bioflocs and can be used by fish and shrimp as an additional source of protein (Azim & Little 2008; Kuhn et al. 2009). Bioflocs, more specifically control aquatic pathogen, because infectious diseases burden the aquaculture industry. These diseases are considered to be the industry's single most important cause of mass mortalities and economic losses. While under certain circumstances antibiotics and other chemical preservatives can provide a useful means of helping to control some bacterial diseases, there are many problems associated with their use. Improper use of chemical preservatives and antibiotics has resulted in the development and spread of (multiple) antibiotic resistance (Defoirdt et al. 2007). As a consequence, there is an urgent need for alternative, more sustainable control techniques.

Quorum sensing by *Vibrio harveyi*.

The Gram-negative bacterium *Vibrio harveyi* is reputed for causing mass mortalities in *Penaeid* shrimp, although they can affect almost all types of aquatic animals, causing disease often referred to as luminescent *Vibriosis* (Phuoc et al. 2009). Virulence of *Vibrio* species has been shown to be linked to quorum sensing, bacterial cell–cell communication with small signal molecules (Defoirdt et al. 2008). Quorum sensing is a mechanism by which bacteria coordinate the expression of certain genes in response to the presence or absence of small signal molecules (Defoirdt et al. 2008). Phenotypes that were found to be controlled by quorum sensing in in vitro-grown luminescent *Vibrios* include bioluminescence and the production of several virulence factors such as a type III secretion system, extracellular toxin, metalloprotease, a siderophore and chitinase (Defoirdt et al. 2010). Moreover, quorum sensing has been shown to regulate in vivo virulence of these bacteria towards different hosts (Defoirdt et al. 2008). Because quorum sensing regulates the virulence of pathogenic bacteria, its

disruption has been proposed as a new anti-infective strategy for aquaculture (Defoirdt et al. 2004). One hundred *Vibrio* strains were isolated from low saline water. Twenty six of them were identified as *V. harveyi*, following various biochemical tests and by the presence of *vhh* gene. The expected size was 234 bps.



Evaluation of AHL production and degradation.

The bacterial strain, *Chromobacterium violaceum* (CV026) as AHL biosensor strain when streaked on Tryptic soya agar. *V. harveyi* of AHL production was streaked, next to CV026. If *V. harveyi* produces AHL diffusible AHL will induce CV026 to purple pigment. It was observed that *V. harveyi* produces AHL. When marine *Bacillus cereus* was streaked it did not produce AHL, hence no purple pigment formed. It was found that twenty five (25) *B. cereus* strains, proved to degrade AHL production.



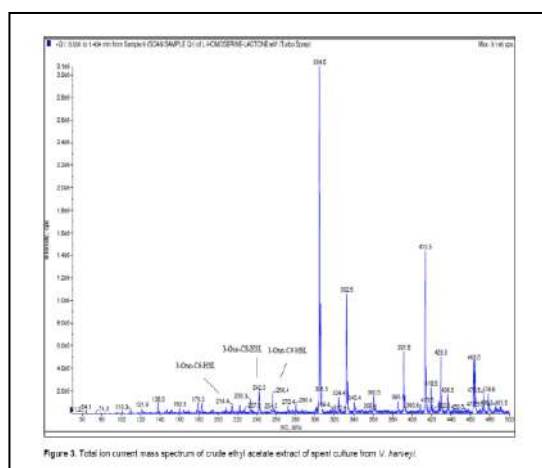
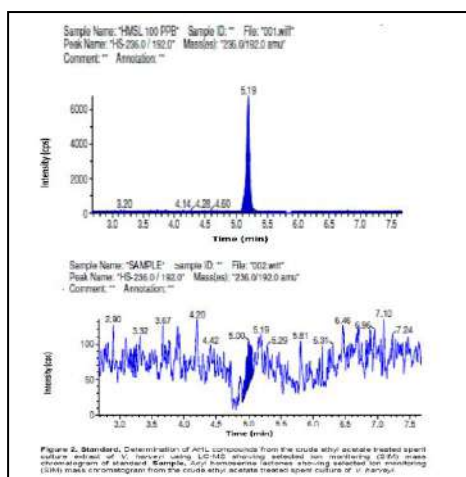
Extraction of N-Acyl homoserine lactone (AHL) compounds (A1 molecules) from *V. harveyi*

One ml of *V. harveyi* was taken as an initial inoculum and added to 1000 ml of LB broth and kept in a shaker incubator (100 rpm, 28°C) for 24 h or until OD reached 1.80. Then, the whole inoculum was aseptically transferred into a sterile centrifuge tubes and centrifuged at 10,000 rpm for 15 min and cell pellets were discarded. The supernatant was filtered through 0.2 µm membrane filter to remove the cell debris. Later, 600 ml of filtrate was mixed with 300 ml of ethyl acetate (2: 1 ratio) and shaker incubated for 10 min. Then, the mixture was allowed to stand for 5 min in a separating funnel to get two immiscible layers (organic layer and aqueous layer). The upper layer was organic layer and the bottom layer was aqueous layer. The organic layer was collected in a sterile container and the remaining aqueous layer was extracted twice as described above. The entire organic layer was pooled and dried by Roto-vapour instrument with a help of vacuum at 30°C. The dried residues were dissolved with 50% of acetonitrile and

water, mixed thoroughly and stored at -20°C for further analysis using LC-MS (Ty A Gould et al., 2006). AHL compound 3 - Oxo hexanoyl homoserine lactone (3-Oxo HSL) was procured from sigma and used as reference standard.

Detection of AHL molecules using Liquid chromatography Mass Spectrometry (LC-MS)

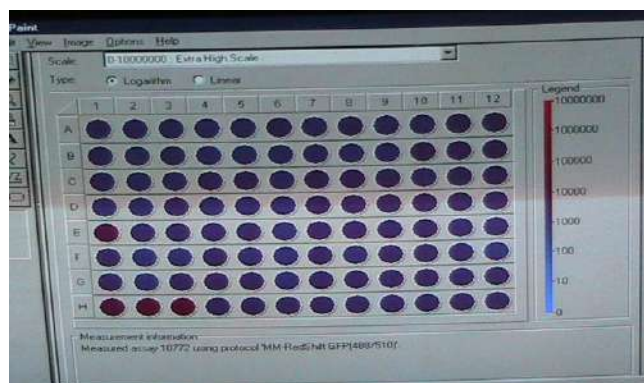
LCMS was performed by “AB Sciex 3200 Q TRAP Linear Ion trap quadrupole” (Liquid chromatography and Mass Spectrometry). The parameters were ion spray voltage of 5500 V, probe temperature of 500°C, curtain gas of 25 units, Gas source of 1 of 40 units to 2 of 40 units, medium units of CAD gas, De-clustering potential of 25 V, entrance potential of 6.50 V, collision energy potential of 16.86 V and collision exit potential of 2V. Nitrogen was used as the collision gas. Multiple reaction monitoring experiments were conducted using the same. The presence of N-acyl homoserine lactones (AHL) compounds from the residues of crude extract and the standard “3-Oxo hexanoyl homoserine lactone” were analyzed separately using Liquid chromatography and mass spectrometry (LC-MS). Different peaks were detected and peaks corresponding to 214.4 m/z were identified as N-(3-oxohexanoyl)-L-homoserine lactones in the sample. Different unknown peaks were also identified.



Extraction of Furanosyl borate diester compounds (A₂ molecules)

V. harveyi was grown in Auto-induction medium (AI) (17.5gl NaCl, 12.3 g MgSO₄, 2.0g L Casomino acids, 1M KH₂PO₄ (10 ml), 20 ml of 50 % glycerol and 0.1 M L-arginine, 10 ml/L) for 16 hr/30°C to the OD of 1.8. The inoculum was diluted to 5000 times in fresh A-2 medium to get 10⁵cfu/ml. One ml of cell free sample was tested for the presence of AI-2, adding to 9 ml of 5000 times diluted cells. This was thoroughly mixed and incubated at 30°C/140 rpm. The bio-luminescence was measured once in 30 min for 12.5 hr by Luminometer. For positive control the reporter strain BB170 (Mutated strain that will respond only to AI-2 molecules) was used to measure the AI-2 molecules. AI-2 production was observed as growth medium dependent. Media composition can greatly influence the luminescence of BB 170 and the test

Vibrio strain sample added as 10% of the total volume. The presence of glucose in AB medium at 1.25 g/l could promote 7 times faster growth causing higher response on BB170. It was found that the signal of the reporter strain was minimal between 5-5.5 hr after inoculation and the interference from exogenous AI-2 of the reporter was less than 1 % of the bioluminescence signal.



Microbial community in bio-flocs

Bacterial populations in biofloc are primarily responsible for water quality maintenance in minimal or zero water exchange systems (intensive systems) viz., heterotrophic ammonia-assimilative and chemoautotrophic nitrifying bacteria. The color changes from green to brown which takes place as the culture progresses due to the transition from a mostly algal-dominant to a bacterial bio-floc-dominant system. The number of bacteria in biofloc ponds can be between 10^6 and 10^9 /ml of floc plug which contains between 10 and 30 mg dry matter making the pond a biotechnological industry. Dominant bacterial species that are present in the bioflocs include *Proteobacterium*, *Bacillus* species and *Actinobacterium*. Besides this, there are some other minor bacterial species such as *Roseobacter* sp. and *Cytophaga* sp. (Zhao et al. 2012).

Effect of glycerol-grown biofloc in controlling *V. harveyi*.

Glycerol-grown biofloc as carbon source were investigated for their antimicrobial and antipathogenic properties against *V. harveyi*. The bioflocs and biofloc supernatants decreased quorum sensing-regulated bioluminescence of *V. harveyi*. Live bioflocs, significantly increased the survival of gnotobiotic brine shrimp (*Artemia franciscana*) larvae challenged against *V. harveyi*. (Crab et al 2010). Biofloc can protect white leg shrimp from *Vibrio parahaemolyticus* bacterial infections.

Biofloc against the growth of other bacteria

Nutritionally, the floc biomass provides a complete source of nutrition as well as various bioactive compounds that are useful for improving the overall welfare indicators of aquatic organisms. Beneficial microbial bacterial floc and its derivative compounds such as

organic acids, poly-hydroxy acetate and polyhydroxy butyrate, could resist the growth of other pathogens, thus serves as a natural probiotic and immunostimulant (Ahmad et al 2017)

Short chain fatty acids as antimicrobial agent.

It was observed that the regular addition of carbon to the culture is known to generate polyhydroxyalkanoate (PHA) accumulating bacteria such as *Alcaligenes eutrophus*, *Azotobacter vinelandii*, *Pseudomonas oleovorans* and others that synthesize PHA granules. These PHAs are polymers of β -hydroxy short chain fatty acids and if degraded in the gut, they could have antibacterial activity similar to short chain fatty acids (SCFAs) or organic acids. The working mechanism of SCFA may be related to the reduction of pH, as well as their ability to dissociate the surrounding milieu (Ricke et al 2003). Apart from inhibiting the growth of pathogenic bacteria by lowering the pH of surrounding milieu, SCFA have also been shown to specifically down-regulate virulence factor expression and positively influence the gut health of animals. SFA is known to inhibit the growth of pathogenic bacteria and they also have been used in commercial diets of terrestrial animals to control pathogens such as *Salmonella* and *E. coli*.

Effect of biofloc on the bacterial ecology and their development

BFT promotes higher densities of total culturable *Vibrios* in the shrimp culture environment. The practice of increasing carbon: nitrogen ratio enhanced growth of heterotrophic bacterial species present in the system. *Vibrio* sp. have long served as models for heterotrophic bacterial processes and are known to efficiently utilized wide spectrum of carbohydrates present in the water (Takemura et al 2014). Most *Vibrio* spp. pathogenic to shrimp such as *V. harveyi* and *V. parahaemolyticus* form green colonies when grown in TCBS agar while those that form yellow colonies were reported to have beneficial effects. The low percentage of green *Vibrio* spp. in the water samples of BFT indicates that the culture system promotes the dominance of yellow *Vibrios* in the rearing water of shrimp. Addition of molasses could have promoted the proliferation of yellow *Vibrios* in BFT since *Vibrio* spp. utilizes sucrose while the green ones do not (Rahman et al 2010). Also, a decreasing trend in the percentage of green *Vibrios* in the BFT tanks was observed in the present study which suggests that there might be a shift in the *Vibrio* species abundance within the system to favour the dominance of other species that may have beneficial roles in the shrimp culture environment. The future of biofloc system is very promissory because it can help to get high productions required to satisfy the growing human population needs. Another advantage in the use of the biofloc system is the decline of the production costs, this is not only beneficial for producers but it could allow the access of more people to this source of animal protein, and improve of human being with scarce economic resources.

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SIGNIFICANCE OF MICROALGAE, ISOLATION AND IDENTIFICATION IN BIOFLOC CULTURE SYSTEM

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Introduction

Biofloc technology is considered as the new blue revolution in aquaculture sector. It is an environmentally friendly aquaculture technique which promotes the retention of waste and its conversion to biofloc as natural food for shrimp and some fishes in the aquaculture system. The technology is applied to a variety of system types and is currently most commonly used for the culture of shrimp and tilapia, although other taxa such as catfish are being explored. The defining features of BFT systems include high animal stocking densities and restricted water exchange. Biofloc consists of microorganisms such as heterotrophic bacteria, algae, fungi, ciliates, flagellates, rotifers, nematodes, metazoans and detritus that conglomerate together and perform symbiotic processes to maintain the water quality, maintain bio-security, support high density of shrimp culture and reduce water exchange in the aquaculture system. Each floc is held together in a loose matrix of mucus that is secreted by bacteria, bound by filamentous microorganisms, or held by electrostatic attraction. A substantial portion of these organisms are contained on and within biofloc particles which can reach a diameter of up to a few mm. These microorganisms are responsible for detoxifying waste products; primarily nutrients excreted by culture animals, and have been shown to provide a repackaging of those nutrients that is available for consumption by animals, thereby lowering feed costs. The biofloc community also includes animals that are grazers of flocs, such as some zooplankton and nematodes. Large bioflocs can be seen with the naked eye, but most are microscopic. Flocs in a typical green water biofloc system are rather large, around 50 to 200 microns, and will settle easily in calm water.

By increasing the C/N ratio in the system, either through the addition of an external carbon source or through using a feed with elevated carbon content, heterotrophic bacterial growth is stimulated or nitrogen uptake through the production of microbial proteins takes place. Although heterotrophic bacteria play the most important role in biofloc formation, the flocs also contain microalgae and these might be important for the nutritional quality of the bioflocs, e.g. through the production of vitamins and fatty acids. The properties of microalgal–bacterial flocs are studied and found that sucrose as a carbon source resulted in good flocs settlement. Hence, it needs to be established to what extent bacteria and microalgae are able to produce bioflocs with the desired nutritional properties. Such microorganisms could be used as an inoculum for the start-up of aquaculture systems with biofloc technology.

Role of microalgae in biofloc

Microalgae play an important key role in the dynamics of biofloc technology aquaculture system. In any biofloc system exposed to sunlight, a dense algal bloom will develop in response to nutrient loading from feeding. Nutrients released from decomposing organic matter are rapidly taken up and stored in algae cells. The rate of algal uptake in biofloc systems is mainly influenced by underwater light intensity. In biofloc systems with a primary dependence on algal uptake, extended periods of cloudy weather can cause spikes of ammonia concentration. The accumulation of biofloc solids shades out algae and limits ammonia uptake. Daily fluctuation in dissolved oxygen concentration and pH, despite intensive aeration, is another characteristic of biofloc systems where algal activity is predominant. Generally, at daily feeding rates less than 300 kg/ha (30 g/m²), algal activity is the major factor controlling water quality.

The interaction between bacteria and microalgae involves different mechanisms, including the production of growth stimulatory or inhibitory compounds, cross-signalling, and the ability of microalgae to act as a vector for specific bacteria. It is expected that well-engineered combinations of microalgae and bacteria might greatly enhance the productivity, efficiency and sustainability of aquaculture. In order to optimally benefit from algae-bacteria interactions, it is important to select the best consortia and thoroughly to investigate the different aspects involved because different combinations of microalgae and bacteria will most probably exhibit different activities. In these systems, a co-culture of heterotrophic bacteria and algae can be grown in flocs under controlled conditions within the culture pond.

Phytoplankton species in biofloc

A diverse group of microalgae include bacillariophyceae, cyanophyceae, chlorophyceae and dinophyceae will present in most of the biofloc culture systems. The commonly occurring species include *Navicula*, *Thalassiosira*, *Nitzschia*, *Amphora*, *Cymbella*, *Cyclotella*, *Coccosinodiscus*, *Oscillatoria*, *Tetraselmis*, and *Chlorella* etc. The biofloc can be modified with the inoculation of desired microalgae. Microalgae with high nutrients composition can be inoculated to the system and enrich the biofloc for better production. The digestion of microalgae (diatoms) by shrimps is studied and it is found that the diatoms can be easily digested by shrimps.

Identification of microalgae in biofloc

Identification of the microscopic biofloc composition can help in better understanding the application of biofloc. From the identification of each class of organisms' function (phytoplankton as primary producer, zooplankton as the algae grazer, bacteria and protozoa as organic matter decomposer) that occurs in the zero water exchange culture system, the interaction happening between the organisms in the biofloc system can be understood. The

microalgal identification can be done by using a standard research microscope. Morphological identification can be done with the help of standard books in microalgal identification. The species level identification may be difficult by the morphologically, so further identification can be done by molecular techniques after isolation of the species.

Microalgal isolation techniques

Often, the first step toward successful isolation is understanding and mimicking the naturally occurring environmental conditions. For coastal marine algae, temperature and salinity are important, and for oceanic (open ocean) phytoplankters, water quality and metal toxicity are additional concerns. The second step toward successful isolation involves the elimination of contaminants, especially those that can out compete the target species. Techniques of dilution, single-cell isolation by micropipette, and agar streaking are widely used, among other methods. The final step requires continued growth upon subculturing. It is not uncommon for the target species to grow in the initial stages of isolation but then die after one or more transfers to fresh culture medium. This often indicates that the culture medium is lacking a particular element or organic compound, which is not immediately manifested. Unfortunately, when this is discovered, it is sometimes too late, because the original sample is gone and the original isolates are dead. Alternatively, the organism may be accumulating wastes that poison its environment, causing death. In nature, these wastes are diluted or metabolized by other organisms (e.g., bacteria). Out of several methods of microalgal isolation; three important methods are explained here: serial dilution, agar streak plating and manipulation.

Serial dilution

Using aseptic technique, dispense 9 ml of specified chemical media (eg f/2 medium) into each of ten test tubes with sterile automatic dispenser or sterile 10 ml pipettes. Label tubes 10^{-1} to 10^{-10} indicating the dilution factor. Aseptically add 1 ml of enriched/fresh sample to the first tube (10^{-1}) and mix gently. Take 1 ml of this dilution and add to the next tube (10^{-2}), mix gently. Repeat this procedure for the remaining tubes (10^{-3} to 10^{-10}). Incubate test-tubes under controlled temperature and light conditions. Temperature and photoperiod should be as close to the natural environment as possible. Light intensity should be slightly lower than the natural environment.

Then examine cultures microscopically after 2-4 weeks by withdrawing a small sample aseptically from each dilution tube. A unialgal culture may grow in one of the higher dilution tubes e.g. 10^{-6} to 10^{-10} . If the number of cells are more observed in test tubes, more dilution has to do till getting pure culture. If tubes contain two or three different species methods like micromanipulation or agar plate streaking can be used to obtain unialgal cultures.

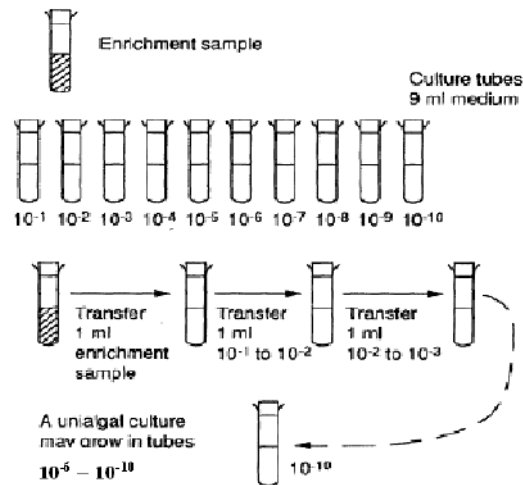


Fig 1: Test tubes in serial dilution

Agar streak plating

This is a suitable method for small species ($<10\mu\text{m}$) or algae that grow well on a substrate. Prepare petri dishes containing growth medium solidified with 1-1.5% agar. The agar should be 1/2-2/3 the depth of the dish. Place 1-2 drops of mixed phytoplankton sample near the periphery of the agar. Sterilize a wire loop in flame. Use the sterile loop to make parallel streaks of the suspension on the agar. Note that there are 16 streaks (4 sets of 4) to be made and the whole surface of the agar plate is used. Cover and seal plate with parafilm. Invert and incubate under low light at constant temperature. Select colonies that are free of other organisms for further isolation. Remove a sample using a sterilized wire loop and place in a drop of sterile culture medium on a glass slide. Check microscopically that the desired species has been isolated and is unialgal. Repeat the streaking procedure with the algal cells from a single colony and again allow colonies to develop. This second streaking reduces the possibility of bacterial contamination and of colonies containing more than one algal species. Transfer selected colonies to liquid or agar medium.

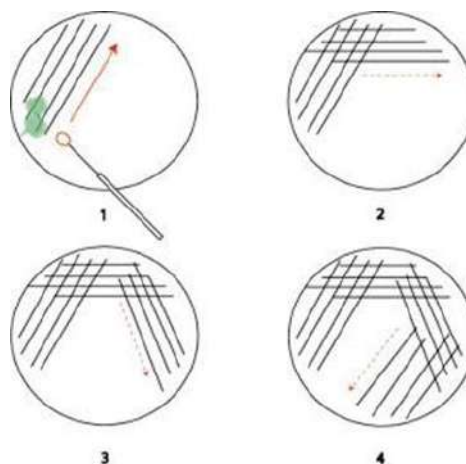


Fig 2: Streak plating with wire loop

Micromanipulation

Micromanipulation is a powerful tool in microalgal isolation. It is usually performed with a pasteur pipette or a glass capillary. A Pasteur pipette can be heated in a flame, extended, and broken. With minimal practice, this technique becomes quick and easy, but the beginner must spend some time practicing before reliable production of micropipettes is achieved. The pipette is held in one hand, and a forceps held in the other hand supports the tip. The pipette is rotated to provide even softening as the pipette warms to the melting point. When the heated area is sufficiently soft, the pipette is removed from the flame and simultaneously pulled to produce a thin tube. If drawn out too quickly, or if drawn out in the flame, then the thin extension breaks or burns through, and the resulting product is unsatisfactory. The goal of micropipette isolation is to pick up a cell from the sample, deposit the cell without damage into a sterile droplet, pick up the cell again, and transfer it to a second sterile droplet. This process is repeated until a single algal cell, free of all other protists, can be confidently placed into culture medium. The process balances two factors: cell damage by excessive handling, which is bad, and clean isolation of a single cell, which is good. For robust organisms, repeated handling can be achieved without damage; however, for delicate organisms, cell damage is an important concern.

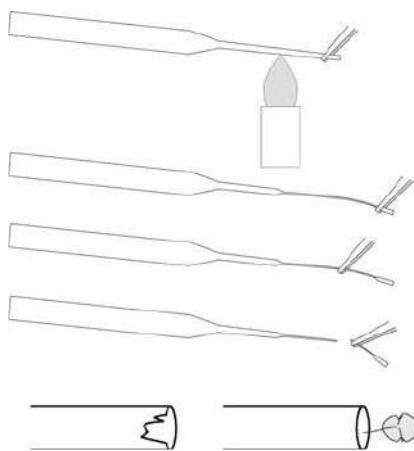


Fig 3: Micromanipulation technique

Phytoplankton Analysis

For quantitative and qualitative analysis of phytoplankton, the water sample collected in 1000ml plastic bottle. Lugol's solution was added as preservatives especially for flagellate and ciliates. It consists of 10g iodine and 20g of potassium iodide dissolved in 200ml of distilled water and 20g of glacial acetic acid. The Lugol's solution is added at the ratio of 1 part in 100 parts of seawater sample. 10ml of Lugol's solution should be added for about 1000ml water sample,

Phytoplankton counting

Water samples were collected from estuary in 500ml bottle, add 2ml of Lugol's solution for preservation and allow settling for 24 hours. On the next day, 450ml of the water (upper layer) was siphoning out carefully and remaining 50ml can be used for plankton counting. Pour 1ml of sample into a Sedgewick raft counting chamber and counting can done by using microscope. There are 500 numbers of blocks in the Sedgewick raft counting chamber and 100 numbers of blocks can be select for counting. Select 20 numbers of blocks from the four corner of Sedgewick raft and one from the center portion in a zig-zag manner and count the number of plankton species individually. Total number of species can be calculated as follows

Number of species in 100 blocks- X

For 500 blocks (1 ml) = 5X

For 50ml= 50* 5* X= 250X

1 20 blocks		2 20 blocks
	3 20 blocks	
4 20 blocks		5 20 blocks

Zooplankton in biofloc

Biofloc act as nutrient retention trap in finfish and crustacean pond culture systems. The composition of phyto and zooplankton of biofloc varies per biofloc maturity time. Zooplankton play a key role in animal's nutrition in culture and have proven benefit in growth rates, in food conversion factor and reduction in costs associated to commercial food. Among the zooplanktons, rotifers, nematodes, crustaceans and protozoans play an important role in nutrient recycle, improve water quality and in nutrition of cultured animals. Proximal composition of zooplankton species such as rotifer (54-60% of raw protein), cladocerans (50-68%) and copepod (70-71%), regarding lipid values in rotifers (3.9 to 13.2%) depending on species, cladoceran (1-2.9%) and copepod (upto 2.6%) (Ray et al., 2010)

The sample was collected by filtering the surface and sub-surface layer of water. The diameter of mouth of net was 30cm and mesh size was 200 micrometer. The samples were fixed and preserved by using 4-5% formaldehyde. The dilution is in the ratio of 1 part formalin and 9 part of fresh water or seawater.

Speed of the boat (V) = 1.5 knot= 30 m/min

Duration of hauling (t) =1 minute

Distance of Hauling (d) = V* t= 30m

If a Plankton net of radius 'r' towed through the water horizontally, the volume of water passing through it would be $\pi r^2 d$ where d is the distance of tow

Volume of water passing through the water = $\pi r^2 h = 2.121 m^3 = 2121$ litre

Zooplankton counting

Suppose 1000 litre of water get filtered through the plankton net and 20ml get collected in the collection chamber. Pour 20ml into a plastic bottle and add 2ml of 4% formalin into it for long term preservation. Pour 1ml of sample into a Sedgewick raft chamber and count the number of zooplankton in 500 blocks (do it for 5 times for getting precise number of zooplankton).

Number of zooplankton in 5 ml of water= X

Number of zooplankton in 1 ml of water = X/5

Number of zooplankton in 20 ml of water= (X/5)* 20

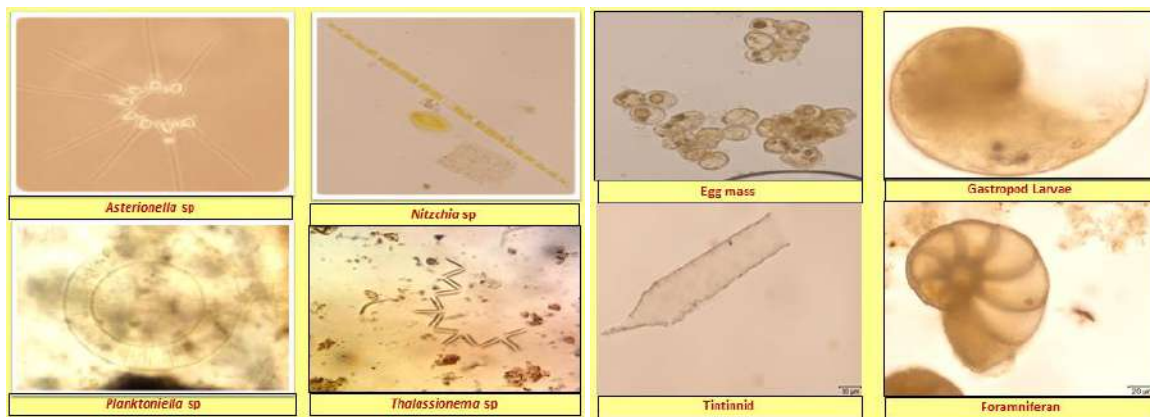
i.e. (X/5)* 20 number of Zooplankton/1000 litre of water

i.e. (X/5)* 20 number of Zooplankton/ m^3 of water

i.e. (X/5)* 20* 100 number of Zooplankton/ $100m^3$ of water

Plankton species in biofloc





Diversity Indices

Species richness index (d)

Species richness is the measure of number of species in the sample.

Pielou Evenness Index (J^1): 0-1

Note:- Value closed to 1 indicate more evenness (For example; suppose there are 100 species in a sample and it consist of 50 species of *Coscinodiscus* and 50 species of *Nitzschia*, then it is evenly distributed and the index value will be closer to 1.

If there are 100 species in a sample and it consist of 80 species of *Coscinodiscus* and 20species of *Nitzschia*. Hence *Coscinodiscus* sp is dominating over *Nitzschia* and the index value may be 0.6. Hence it is less evenly distributed compare to previous one.

If there are 100 species in a sample and it consist of 60 sp of *Coscinodiscus* and 40 sp of *Nitzschia*, it is not evenly distributed and *Coscinodiscus* is dominating over *Nitzschia* and the index value may be 0.7. Hence it is more evenly distributed compare to previous one (80-20) and less compare to first one (50-50).

Shannon's diversity index (H^1)

1- Indicate less diverse

1-3- indicate Moderate diverse

>3- indicate High biodiversity

Simpson diversity Index ($1-\lambda$) - 0-1

1 - Infinite diversity

0 - No diversity

~1- More diverse

~0 - Less diverse

Conclusion

A variety of beneficial features can be attributed to biofloc technology, from water quality control to *in situ* feed production and some possible extra features. Biofloc technology offers aquaculture a sustainable tool to simultaneously address its environmental, social and economic issues concurrent with its growth. The basics of the technology is there, but its further development, fine-tuning and implementation will need further research and development from the present and future generation of researchers, farmers and consumers to make this technique a keystone of future sustainable aquaculture. The exact manipulation of biofloc with potential microalgae will enhance the production. For further development of biofloc technology in that line more research has to be done for the interaction of microalgae with other components of biofloc and their influence in the system.

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AQUAMIMICRY: AN INNOVATIVE ORGANIC APPROACH

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Introduction

Demand of environment friendly farming resulted in introduction of biofloc and biomimicry kind of technology which is developed by supplementation of external carbon sources leading to the colonization of microbial/microalgal population. This will convert the harmful sediments into consumable nutrient source which reduce the toxic deposition and also considerably reduced the need of water exchange. This is an ecofriendly farming followed in many places and successfully resulting bio secured shrimp production. More recently organic shrimp farming is also a different production strategy called as Aquamimicry, has received a great attention worldwide mainly because of the growing market demand and high commercial value attained by the product. In general shrimp farming is carried out by artificial or commercial feed management's methods. Organic shrimp farming has been applied successfully and effectively in shrimp farming. Organic shrimp farming is a rising alternative modern method towards more ecofriendly aquaculture production system. This technology was developed to create economical and environmental benefits via reduced artificial feed supply. This technology may otherwise called as aquamimicry. It means the connection of aquatic biology and technology mimicking the nature of aquatic ecosystems to create living organisms for the wellbeing development of aquatic animals, which is believed to effectively provide a sustainable revival to shrimp farming industry. Aquamimicry technology adopted to provide natural estuarine environment by promoting and balancing the natural planktonic community's growth.

Aquamimicry

Aquamimicry simulate natural pond condition by developing beneficial phyto and zooplankton blooms as supplemental nutrition to the farmed shrimp and beneficial microbes to maintain water quality using carbon source such as rice bran and wheat along with probiotics. Aquamimicry concept is similar to biofloc technology but differing in the following ways;

- (i) Reduced carbon sources (especially rice bran) addition and also its quantity not depend on nitrogen ratio.
- (ii) Also sedimentation of suspended bioflocs are frequently removed and reused for other purposes like manure, animal feed etc.

With these alterations from biofloc, the culture environment preferably mimics natural estuarine environment with balanced water quality by acquired planktons. So the use of feed and also water exchange can be reduced. The carbon sources serves as a feed for planktonic growth which served as feed for microbes and farmed shrimps. The main

advantages of this technology is

1. More sustainable than conventional method
2. Shrimp develops good immunity and health
3. Decreasing feed conversion ratio
4. Minimal water exchange is required
5. Chance of disease outbreak is reduced
6. Improve overall nutrition of farmed shrimp
7. Reduce stress to the animals and minimize favorable condition to harmful organism.

Feed used in organic shrimp production shall be produced from natural ingredients. The feed management can reduce the feed conversion ratio and environmental problem. Feed for organic shrimp production can be divided into two groups namely natural feed and commercial feed. Natural feed means live animals like zooplanktons, artemia and phytoplanktons in the water resources used for shrimp farming. Shrimp do not feed directly on the phytoplankton. They feed on the small animals that eat the phytoplankton or on bacteria that grow on the dead phytoplankton cells which accumulate on the bottom. Zooplanktons are the natural food of the larval stages of many aquaculture commodities. They are the animal component of plankton and are often referred as herbivores or grazers, feeding heavily on phytoplankton. In aquaculture seed production, rotifers and cladocerans are the preferred prey of fish and crustacean larvae due to their suitable size. There is a high chance of success in larval rearing if there is a good correlation of prey size to the gape size of fish larvae. The culture of zooplankton is considered as the second order of live food production in aquaculture systems. The success of a multi-species hatchery mainly depends on the continuous availability of live food of high nutritional value.

Hands on method

Aquamimicry technology particularly aimed to promote the growth of copepods and most suitable carbon source identified is rice bran fermented with suitable probiotic bacteria. The process could be initiated by adding probiotic bacteria to grain rice bran and allowed to ferment 24 h. Fermented rice bran at the rate of 500-1000 Kg / ha will be supplemented to the pond. This will promote the dominant growth of copepods within a week time and after that the post larvae will be stocked (10-20 individuals/ m²). The fermented rice bran at the rate of 1 ppm will be added daily throughout the culture cycle for the purpose of maintaining the zooplankton as a feed for especially during early stocking and also for creating minor biofloc (< 25 mL/L as measured by an Imhoff cone). Later for feeding, fermented soy bean meal will be used which completely reduced the feed cost. Additional development of biofloc will be very much helpful to fulfill the nutritional requirement and thus reduced the feed cost. (Chakravarty et al., 2018; Nicholas and Romano 2017; Romano and Kumar 2017)

Aquamimicry shrimp farming protocol

First Step: Pond preparation

- Fill the pond with filtered seawater using filter bag (200-300 μ)
- Add probiotics (*Bacillus* sp)
- Gentle dragging through the bottom of the pond for one week in earthen pond to enhance soil mixing with added probiotics and minimize biofilm development (harmful to shrimp). In lined pond, heavy rope can be used to prevent tearing of sheet.
- In order to remove unwanted aquatic weeds, tea seed cake can be applied at the rate of 20ppm along with fermented rice bran or wheat bran (without husk) at 50-100ppm to develop zooplankton blooms.
- Heavy aeration is must for proper mixing of nutrients and probiotics and reduces tea seed cake level.

Second step: Application of carbon source

- Add rice bran/ wheat bran (without husk) mixed with water at a ratio of 1:5 to 1:10 and probiotics under aeration for 24hr. If the bran is powdered completely, entire mixture can be added slowly to the pond. If crumbled, upper layer of milk/juice added to the pond.
- pH of the incubation water should be 6-7

Third step: Stocking of Post larvae

- Post larvae can be stocked at a stocking density of 30-40 nos/m² (Post larvae 12-15)
- Amount of ferment added depending on turbidity of water (30-40 cm) i.e. 1ppm for extensive system and 2-4ppm for intensive system
- Analyze water quality parameters on daily basis
- Gentle dragging should be done in 15 days intervals after stocking to minimize biofilm formation
- Additional probiotics should be added on every month during growout period to maintain water quality and promote plankton growth
- In an intensive culture system, excess sediment should be removed using central drainage system to sedimentation pond after 2hr of each feeding interval
- Sedimentation pond (Normally 4m deep at center and 2m at edges) can be stocked with fish species such as milkfish and cat fish at low densities which can feed the plankton and detritus provide alternate source of income to the farmers. Sediments from growout pond encourage production of worms and can utilize by the animals.
- Water overflows from sedimentation ponds to another pond to increase retention time and act as biofilter in which fish species like tilapia can be stocked at low densities. From here, water can be overflows to grow out pond with little nitrogenous waste. Sedimentation should be cleaned in every 3 year intervals.

Fourth step: Post harvest

After the harvest, pond is free from black soil, accumulated sediments due to regular removal of sediments. Therefore pond is ready for next production cycle by addition of fermented rice bran and probiotics.

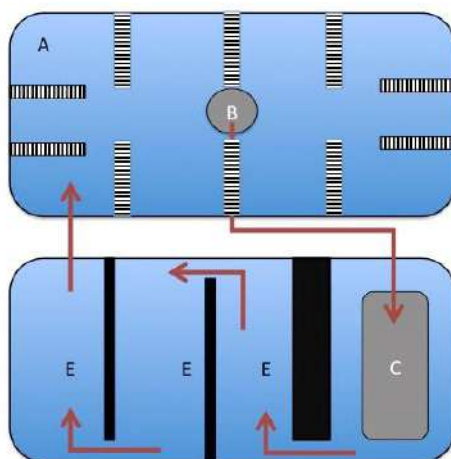


Fig 1. General layout of aquamimicry concept adopted in Thailand

A- Grow out pond with eight long arm paddle wheel;

B- Sump; C- Sedimentation pond; D-Biofilter pond

Aquamimicry- Adoption by an innovative farmer from Andhra Pradesh

Ganesh Mokkaapati, a farmer from Vijayawada, Andhra Pradesh adopted aquamimicry based shrimp farming with a stocking density of 40 PL/m². Long arm aerators with plus shape design were used to keep the pond bottom in aerobic conditions. Before starting new cycle, he analyzed soil pH, ammoniacal and nitrate form of nitrogen and treated soil with good soil probiotics. Fermented rice bran helped in developing good amount of zooplankton. Pond water treated with probiotics and fermented rice bran turns the colour of the water to golden brown and maintains pH stable throughout the culture period by adding 5-10 ppm fermented rice bran and also enhance the development of planktons. Timely removal of sludge kept the bottom clean which prevent production of toxic gases like ammonia, nitrite to the system. Alternate natural feed like fermented soya with fish sauce for two meals and commercial pellet for two meals were applied to reduce cost of production. ICAR CIBA helped the farmer from monitoring the water quality, estimation of greenhouse gases throughout the culture period ensuring that the aquamimicry farming is environmentally sustainable. With a stocking density of 40 nos/m², the production obtained was 5.53 tons with a survival rate of 94%. The cost of production is Rs 199/ Kg whereas the farm gate price was Rs 330/ Kg.

Conclusion

Aquamimicry is a revolutionary concept to effectively provide a sustainable rival to the shrimp farming industry. It is an intersection of aquatic technology synergistically working

together in mimicking the nature of aquatic ecosystems to create live food organism for the well-being of aquatic animals. Shrimp produced this technology are red in color when cooked, likely from the consumption of natural foods that contain pigments (astaxanthin, amino acids and fatty acids, such as omega-3 fatty acids) which will add the marketing value as like “Organic shrimp” (Romano and Kumar, 2017). Organic marine shrimp farming means farm management practices for shrimp that introduces specific requirement to an organic standard. They are based on the holistic agriculture management, ecofriendly and sustaining biodiversity. All input materials shall be natural products, avoid using synthetic products and any genetically modified organisms. In order to maintain the specific qualities of organic status, this management shall be practiced throughout the production chain.

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ECONOMICS OF BIO FLOC BASED FARMING

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Biofloc technology

Biofloc technology became more and more popular with advent of farming of Pacific white shrimp, *Penaeus vannamei*. The technology initiated by Yoram Avnimelech (2000) in Israel and was initially implemented commercially in Belize by Belize. It also has been applied with success in shrimp farming in Indonesia, Malaysia, Australia etc.

The combination of two technologies, partial harvesting and biofloc, has been studied in northern Sumatra, Indonesia (Nyan Taw 2008 et. al). The system has been successfully incorporated in bio secure modular culture system (Nyan Taw, 2011). With emerging viral problems and rising costs for energy, biofloc technology appears to be an answer for sustainable production at lower cost. The technology has applied also in super-intensive raceways to produce more than 9 kg shrimp/ m³. The raceway applications have supported nursery and grow out to shrimp brood stock rearing and selection of family lines.

In ICAR-CIBA, a number of studies using biofloc as a protein source in shrimp feeds were conducted. In any aquaculture business as defined by economics-savings are also considered as profit. Savings such as from feed, time, energy, stability and sustainability can be calculated as profit.

Biofloc system

For optimized, sustainable commercial bio floc shrimp culture, high-density polyethylene- or concrete-lined ponds are basic requirements. High stocking densities of 130-150 post larvae/m² and high aeration rates of 28-32 hp/ha is also essential. Paddlewheel aerators are placed in ponds to keep dissolved-oxygen levels high and guide sludge toward the central areas of ponds. The sludge can then be siphoned out periodically when needed. The aerators help suspend the bioflocs in the pond water – a main requirement for maximizing the potential of microbial processes in shrimp culture ponds. The suspended biofloc is also readily available as feed for shrimp. Pelleted grain and molasses are used to sustain carbon: nitrogen ratios above 15.

Generally, incoming water is screened to prevent larval crustaceans (especially crabs) from entering reservoirs and culture ponds. The most important factor is to make sure the screening, chemical treatment and aging process are efficiently used before stocking ponds with shrimp. Only specific pathogen-free post larvae should be stocked. Once ponds are stocked, a major factor to control is biofloc volume. Biofloc volumes need to be maintained below 15 ml/L. Green or brown water is acceptable, but black water is an indicator of

abnormal conditions. Grain pellets and molasses supply sources of carbon as needed. Generally, grain pellet applications vary from 15 to 20% of the total feed used during operations. Molasses can be applied two or three times. Dissolved oxygen needs to be monitored as frequently as possible to keep levels higher than 4mg/L. Especially in biofloc systems, aerators need to be constantly monitored for malfunctions and repaired or replaced without delay.

In sum biofloc system can be summarised as:

- High stocking density - over 130 – 150 PL10/m²
- High aeration – 28 to 32 HP/ha PWAs
- Paddle wheel position in ponds (control biofloc & sludge by siphoning)
- Biofloc control at <15ml/L
- HDPE / Concrete lined ponds
- Grain(pellet)
- Carbohydrate
- 8 C&N ratio >15
- Expected production 20–25 MT/ha/crop with 18-20 gms shrimp.

Culture performance

A comparison of the expected cost savings and culture performance of the biofloc system to a traditional autotrophic system:

- Shrimp grow faster and yield a larger harvest in biofloc systems.
- Feed conversion is better with biofloc, so feed costs are lower.
- Days of culture is reduced and hence more cycles can be taken
- Less water is exchanged with biofloc technology, less pumping costs and the pond systems are more stable than in autotrophic culture. Greater output with bioflocs also improves energy use.

In addition to intensive fish and shrimp culture, biofloc technology has been applied in super-intensive raceways to produce over 9 kg shrimp/ m³. The raceway applications have supported nursery, growout and shrimp broodstock operations, as well as selection of family lines. Though feed sales may reduce quantitatively it will be sustained in the long run

Economic perspectives

Since, biofloc technology option needs to be economically evaluated in farmers' angle so that the technology can be adopted and up scaled if it is economically advantageous over auto trophic

system. Data on costs and returns obtained from trials conducted by ICAR-CIBA are presented in Table.1.

It can be noted from the Table.1, the fixed input items are approximately Rs.2.72 lakhs. The plastic items may need replacement after two years which is taken care in accounting for fixed investments in a five year cash flow. The variable inputs cost about Rs.5.6 lakhs per cycle. Each year optimally 6cycles can be taken in normal circumstances. Hence, year wise costs and returns are calculated on assumption of 6 cycles per year.

The sensitivity analysis in Table.1 portrays the results of financial analyses of the production data. Apart from normal, best and worst cases were created based on sale price of nursery grown larvae and survival rate in percent, the two critical parameters in BFT. The IRR is the largest in best case scenario of 90% survival and sale price of Rs.1/piece, which is not very difficult to achieve in professionally managed hatcheries. Even in the worst case scenario of 80% survival and sale price of Rs.0.90/piece, the entrepreneur will get an IRR of 39% which is reasonable.

Table. 1 Cost of production of Nursery grown shrimp seeds under biofloc culture technology

S.No	Costs (Rs.)	Year 0	I cycle	Year 1 (6 cycles)	Year 2	Year 3	Year 4	Year 5
A	Fixed Cost							
1	100 ton lined nursery pond with central drainage	80000				36000		
2	Aerator and Aeration system	30000				13500		
3	Lab for water quality, microbiology + DO meter	20000				9000		
4	Biosecurity nets and fixers	25000				25000		
5	Nets , Pipes etc + drain pit	25000				25000		
6	Plastic item(Mug, Feeding tray, Basket)	12000				12000		
7	Storage room, blower room, staff quarters, Wash room, OHT	50000						
8	Generator, cabling, lighting, pumps	20000				6000		
9	Kitchen and mess	10000						
	Total Fixed cost	272000				126500		
B	Operational cost							

(I) Seed @ 0.40 Rs/per seed (Including transportation) SD @ 7000 nos/cu m		280000	1680000	1680000	1680000	1680000	1680000
(II) Seed testing x 3 times		9000	54000	54000	54000	54000	54000
(Ili) Disinfectant (Bleaching, NAOH)		4000	24000	24000	24000	24000	24000
(IV) Feed @ FCR 0.8 (150 kg @100/-)		15000	90000	90000	90000	90000	90000
(V) Carbon source-900 @ 25/-		22500	135000	135000	135000	135000	135000
(VII) Mineral and Other supplements		8000	48000	48000	48000	48000	48000
(V) Diesel @ 54 Rs/1 lit./Power for Water pumping		7425	44550	44550	44550	44550	44550
Labour Cost 24 hour duty 6 labour per 4 tank x 15,000 Rs incl food stay incentive		45000	270000	270000	270000	270000	270000
Technician 2 x30,000 incl food incentive etc		30000	180000	180000	180000	180000	180000
Management cost incl. seed selection, accounts, purchase, Training, sales, travel		15000	90000	90000	90000	90000	90000

Table.2 Profit of production of Nursery grown shrimp seeds under biofloc culture technology

S.No	Costs in Rs.	Year 0	I cycle	Year 1 (6 cycles)	Year 2	Year 3	Year 4	Year 5
	Labour during stocking , harvest and transportation plus ice, oxygen etc		7500	45000	45000	45000	45000	45000
	Testing Costs		3000	18000	18000	18000	18000	18000
	EMI Principal+ Interest@14% for 7 lakh loan (fixed and 1 cycle VC)			76514	229542	229542	229542	153028
	Variable costs		443425	2755064	2908092	3034592	2908092	2831578
	Total Cost A+B	272000	443425					
	Revenue		560000					
Normal	Gross Returns Nursery grown shrimp seed sold @ 1.00 per piece (SR 80%)	0	560000	3360000	3360000	3360000	3360000	3360000
	Total expenditure Expected per annum 6 cycles	272000	443425	2755064	2908092	3034592	2908092	2831578
	Net profit /Profit (D-C)	-272000	116575	604936	451908	325408	451908	528422
	B/C Ratio		1.263	1.220	1.155	1.107	1.155	1.187
	Rate of Return	111%						
Sensitivity analysis for Price and Production changes								

Best	Gross Returns Nursery grown shrimp seed sold @ 1.00 per piece (SR 90%)	0	630000	3780000	3780000	3780000	3780000	3780000
	Total expenditure Expected per annum 6 cycles	0	443425	2755064	2908092	3034592	2908092	2831578
	Net profit /Profit (D-C)	-272000	186575	1024936	871908	745408	871908	948422
	B/C Ratio		1.421	1.372	1.300	1.246	1.300	1.335
	Rate of Return	172%						
Worst	Gross Returns Nursery grown shrimp seed sold @ 0.90 per piece (SR 80%)	0	504000	3024000	3024000	3024000	3024000	3024000
	Total expenditure Expected per annum 6 cycles	0	443425	2755064	2908092	3034592	2908092	2831578
	Net profit /Profit (D-C)	-272000	60575	268936	115908	-10592	115908	192422
	B/C Ratio		1.136607	1.097615155	1.03986	0.99651	1.03986	1.06796
	Rate of Return	39%						

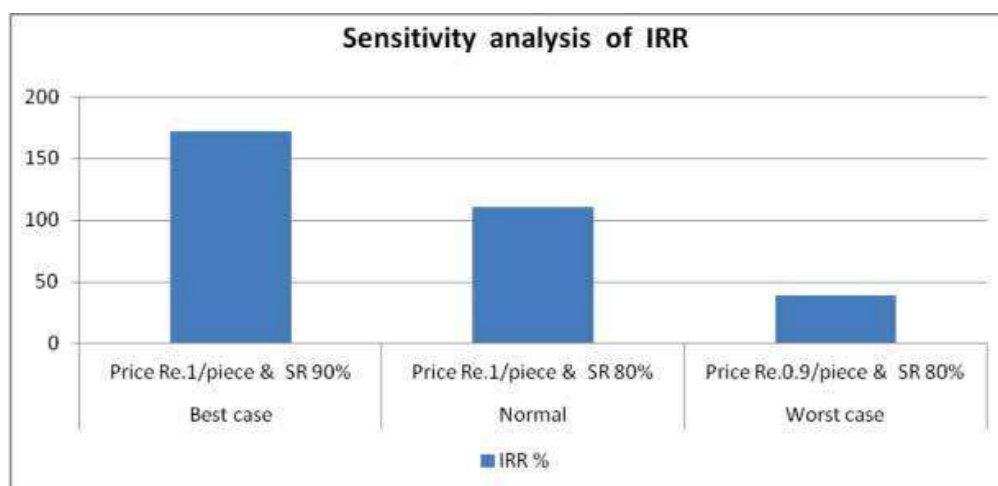


Fig.1. Sensitivity of Internal Rate of Return under optimistic, Normal and Pessimistic scenarios

Conclusion

In any aquaculture business, savings from efficient use of feed, time, energy, system stability and sustainability can improve profits. It seems biofloc technology has these properties. With emerging viral problems and rising costs for energy, biofloc technology with bio secure modular systems may be an answer for more efficient, sustainable, profitable aquaculture production. Related to biofloc meal and its perspectives, an earlier study detected initial estimates of cost for producing a metric ton of biofloc meal is approximately \$400 to \$1000. The same authors cited that global soy meal market varied approximately from \$375 to \$550/metric ton from January 2008 through May 2009. During the same time period, fish meal varied approximately from \$1000 to \$1225, suggesting feasibility on replacement of either soybean and/or fish meal by biofloc meal. Moreover, generated from a process that cleans aquaculture effluents biofloc meal production avoids discharge of waste water and excessive damage to natural habitats. This ingredient seems to be free of deleterious levels of mycotoxins, antinutritional factors and other constituents that limit its use in aqua feeds. Large-scale production of biofloc meal for use in aquaculture could result in environmental benefits to marine and coastal ecosystems, as the need for wild fish as an aqua feed ingredient is reduced. Sensorial quality of BFT products is also an important issue. BFT may bring higher profit if fresh non-frozen shrimp/fish is sold to near-by market, mainly at inland locations. These advantages certainly should be more explored and niche markets achieved, contributing to social sustainability.

PRACTICALS

1. ANALYSIS OF BRACKISHWATER

Total Settleable solids

Principle: This is a portion of organic and inorganic solids that settles in 1 h in an Imhoff cone and is measured in terms of ml/l.

Procedure: Shake the water sample vigorously and pour 1 litre water into Imhoff cone graduated at the lower end and leave it for 1 h. Measure the quantity of settleable solids in ml/l.

Total Suspended Solids (TSS) and Total Dissolved Solids (TDS)

Principle: A well-mixed sample is filtered through a weighed standard glass fibre filter disc or Gooch crucible made of porcelain and the residues retained on the filter is dried to constant weight at 103°C to 105°C. The increase in weight of filter represents the total suspended solids. For total dissolved solids, the filtrate is evaporated to dryness in a weighed dish and dried to constant weight. The increase in dish weight represents the total dissolved solids.

Procedure: Wash filter disc with three successive 20 ml volumes of distilled water using vacuum. Continue suction to remove all traces of water. Filter a measured volume of well mixed sample through the glassfibre filter to Gooch crucible. Wash with three successive 10ml volumes of distilled water allowing complete drainage between washings and continue suction for about 3 minutes after filtration is complete. Transfer filtrate to a weighed evaporating dish for measurement of total dissolved solids.

Total suspended solids

Dry filter disc/crucible containing residues for at least 1h at 103°C-105°C in an oven. Cool in a dessicator and weigh. Repeat the cycle of drying, cooling, desiccating and weighing until a constant is obtained.

$$\text{TSS (mg/l)} = \frac{(A-B) \times 1000}{\text{Sample volume (ml)}}$$

A = Weight of filter or crucible + dried residue (mg)

B = Weight of filter or crucible (mg)

Total Dissolved Solids

Evaporate the filtrate in dish to dryness on a steam bath. Dry for atleast 1 h in an oven at 180°C, cool in a desiccator and weigh. Repeat drying, cooling, desiccating and weighing until a constant weight is obtained.

$$(A-B) \times 1000$$

$$\text{Total dissolved solids (mg/l)} = \frac{\text{-----}}{\text{Sample volume (ml)}}$$

A = Weight of dried residues + dish (mg)

B = Weight of dish (mg)

Dissolved Oxygen

Principle: DO can be determined by Winkler's method. In this method divalent manganese solution, followed by strong alkali, is added to the sample. Any dissolved oxygen rapidly oxidizes an equivalent amount of divalent manganese to basic hydroxides of higher valency states. When the solution is acidified in presence of iodide ions, the oxidized manganese ions again reverts to divalent state and iodine, equivalent to the original dissolved oxygen content of the water, is liberated. This iodine is titrated with standardized thiosulphate solution.

Reagents

- **Winkler A solution** (Manganous sulphate): dissolve 480 g $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ or 400 g of $\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$ or 365 g of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ in distilled water and make up the volume to 1 litre.
- **Winkler B solution** (alkaline iodide): Dissolve 500 g of sodium hydroxide and 300 g of potassium iodide in 900 ml of distilled water and make up the volume to 1 litre.
- **Standard thiosulphate solution** (0.025 N): To prepare 0.1 N stock solution of sodium thiosulphate, dissolve 24.82 g of crystalline $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ and 4.0 g of borax as a preservative in 700 ml of distilled water and make up the volume to 1 litre. Standardize the strength of this solution to exactly 0.1N by titrating against 0.1N potassium dichromate. To make 0.025N thiosolution, dilute 125 ml of this standardized stock solution (0.1 N) to 500ml
- **Concentrated sulphuric acid.**
- **N potassium dichromate:** Dissolved 4.904 g of dried and crystalline $\text{K}_2\text{Cr}_2\text{O}_7$ in 1 litre of distilled water.
- **Starch solution (0.2%):** Add 2.0 g starch and 30 ml 20% NaOH solution in 350 ml of distilled water. Stir until thick, almost clear solution is obtained. Neutralise the alkali with HCl and acidify with 1 ml of glacial acetic acid. Finally dilute the solution to 1 litre with distilled water.

Procedure: Collect the water sample in stoppered BOD bottle and add immediately 1 ml of manganoussulphate reagent with a pipette followed at once by 1.0 ml of alkaline iodide solution.

Restopper the bottle immediately and mix the contents thoroughly by shaking to develop a flocculent precipitate. No air bubble should be trapped in the bottle. Add concentrated sulphuric acid (about 1 ml) to dissolve the precipitate. Transfer 50 ml of dissolved solution into a conical flask. Titrate at once with 0.025 N standard thiosulphate solution until a very pale straw colour remains. Add starch (about 5 ml) indicator and continue the titration until the blue colour is just discharged. Solution should remain colourless for at least 20 seconds at the endpoint.

Calculation

$$\text{DO (ppm)} = \frac{8000 N \times V_1}{V_2}$$

V_1 = Volume (in ml) of $\text{Na}_2\text{S}_2\text{O}_3$ of normality N required for titration

V_2 = volume of water sample titrated.

If $N = 0.025\text{N}$ and $V_2 = 50\text{ ml}$ then $\text{DO (ppm)} = V_1 \times 4$

Chemical Oxygen Demand

Principle: COD is a measure of organic matter and represents the amount of oxygen required to oxidize the organic matter by strong oxidizing chemicals (potassium dichromate) under acidic condition. The excess dichromate is titrated with standard ferrous ammonium sulphate using ferroin as an indicator. Mercuric sulphate is added to complex the chlorides, thereby effectively eliminating the chlorides interference.

Reagents

- **0.05 N Potassium dichromate:** Dissolve 2.452 g dried, crystalline $\text{K}_2\text{Cr}_2\text{O}_7$ in distilled water and make up the volume to 1 litre.
- **0.05 N Ferrous ammonium sulphate (FAS):** Dissolve 19.61 g of $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ in 800 ml of distilled water containing 1 ml of conc. sulphuric acid and make up the volume to 1 litre.
- Mercuric sulphate
- **Ferroin indicator:** Dissolve 1.888g of 1:10 phenanthroline monohydrate and 0.70g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ in 100 ml of distilled water.

Procedure: Pipette out 20ml of water sample into a 125ml Erlenmeyer flask. Add exactly 10 ml of 0.05 N $\text{K}_2\text{Cr}_2\text{O}_7$ solution to the flask. Add 200 mg HgSO_4 for each 1000 mg per litre of chloride ($\text{HgSO}_4 : \text{Cl}^- : 10 : 1$). Swirl until the HgSO_4 is dissolved. Add carefully 30 ml of conc. H_2SO_4 . Cover the flask with watch glass and allow to stand for 30 min. Add 15 ml of distilled water and 3 drops of ferroin indicator and titrate the whole reaction mixture with FAS of same normality. Prepare blank with 20ml distilled water and repeat the same procedure.

Calculation

$$\text{COD mg/l} = \frac{(B-S) \times N \times 8000}{V}$$

B = Titre value for Blank in ml

S = Titre value for sample in ml

N = Normality of FAS

V = Volume of sample in ml

Biochemical Oxygen Demand

Principle: The sample of water or appropriate dilution is incubated for 5 days at 20°C in the dark. The reduction in DO concentration during the incubation period yields a measure of the BOD.

Reagents: Use all the reagents required for the determination of DO.

Procedure: Collect three water samples from one site into BOD bottle following the procedure for DO. Determine the DO level in one of these samples, whilst the remaining two samples are firmly stoppered and placed in an incubator at 20° C in the dark for 5 days. At the end of this time, the DO level is determined by the usual Winkler's titration.

In heavily polluted samples, it is necessary to dilute the sample with a known amount of clean, air saturated water, so as to obtain required dilution (almost 50%). Siphon out the mixed sample into two sets of specially designed BOD bottles, one set for incubation and the other for determination of initial DO.

Calculation

Initial DO = D_o ppm

Final DO = D ppm

Reduction in DO = $D_o - D = D_{r1}$ ppm

Dilution water initial DO = D_1 ppm

Final DO = D_2 ppm

Reduction in DO = $D_1 - D_2 = D_{r2}$ ppm

Therefore reduction due to sample = $D_{r1} - D_{r2}$ ppm = D_s

BOD (ppm) = $D_s \times \text{Dilution factor}$

Alkalinity

Principle: It can be measured by titrating the water sample with a standard acid using methyl orange.

Reagents:

- **0.02 N Sulphuric Acid:** Dilute 30 ml of concentrated H_2SO_4 to 1 litre with distilled water to get approximately 1N stock solution. To make 0.02N H_2SO_4 , take 20 ml of this stock solution and dilute to 1 litre with distilled water. Standardise this solution against 0.02N sodium carbonate using methyl orange as in indicator.
- **0.02 N Sodium carbonate:** Dissolve 5.3 g anhydrous sodium carbonate in 1 litre distilled water. Dilute 50 ml of this solution to 250 ml to get 0.02 N sodium carbonate.
- **Methyl orange indicator:** Dissolve 0.05 g reagent in 100 ml of distilled water.

Procedure: Add 2 drops of methyl orange indicator to 50 ml of water sample. If the sample remains colorless, no alkalinity is there. If it is yellow, titrate with 0.02N H_2SO_4 till the color turns taint orange.

Calculation

Total alkalinity (ppm of CaCO_3) = volume of 0.02 N H_2SO_4 required for titration x 20

Ammonia-N

Principle: Water sample is treated in an alkaline citrate medium with sodium hypochlorite and phenol in the presence of sodium nitroprusside which acts as a catalyzer. The blue indo phenol colour formed with ammonia is measured spectrophotometrically.

Reagents

- **De-ionised water**
- **Phenol solution:** Dissolve 20 g of analytical grade phenol in 200 ml of 95% v/v ethyl alcohol.
- **Sodium nitroprusside solution:** Dissolve 1.0 g of sodium nitroprusside, $\text{Na}_2\text{Fe}(\text{CN})_5\text{NO} \cdot 2\text{H}_2\text{O}$, in 200 ml of de-ionised water. Store in a dark glass bottle. The solution is stable for at least a month.
- **Alkaline reagent:** Dissolve 100 g of sodium citrate and 5 g of sodium hydroxide in 500 ml of de-ionised water. The solution is stable indefinitely
- **Sodium hypochlorite solution**
- **Oxidising solution:** Mix 100 ml of reagent 4 and 25 ml of reagent 5. Prepare fresh every day.

Procedure: Add 50 ml of seawater to an Erlenmeyer flask from 50 ml measuring cylinder. Add 2 ml of phenol solution, swirl to mix and then add in sequence 2ml of nitroprusside 5ml of oxidizing solution. Mix after each addition by swirling the flasks. Cover the flasks with aluminum foil to lessen the contamination by atmospheric ammonia and allow the flasks to stand at room temperature for 1 hr in dark. The colour is stable for about 24 hr after the reaction period. Read the absorbance at 640 nm in a spectrophotometer against blank or distilled water using 10 cm cell. Carry out the method exactly as described above for blank also using 50 ml of de-ionized water.

Calculation: Calculate the ammonia concentration by using calibration curve.

Standard curve: Dissolve 0.9433 g of analytical reagent quality ammonium sulphate in 950 ml of distilled water. Add 1ml of chloroform and make up the volume to 1 litre. Store in refrigerator, sheltered from strong light. This solution contains 200ppm and is stable for many months if well stoppered. Prepare a series of standard solutions from this stock solution and carry out the method exactly as described above. After colour development, measure absorbance at 640 nm and prepare a calibration curve from the absorbance of a series of standards.

Nitrite-N

Principle: The nitrite in water is allowed to react with sulfanilamide in an acid solution. The resulting diazo compound is reacted with NED and forms a highly coloured azo dye.

Reagents

- **Sulfanilamide solution:** Dissolve 5.0 g of sulfanilamide in a mixture of 50 ml of conc. HCl and about 300 ml of distilled water. Dilute to 500 ml with distilled water. The solution is stable for many months.
- **NED (N-(1-naphthyl) ethylene diaminedihydrochloride solution):** Dissolve 0.5 g of the dihydrochloride in 500 ml of distilled water. Store the solution in a dark bottle. The solution should be renewed once a month or directly a strong brown colouration develops.
- **Standard nitrite:** Dissolve 1.064 g anhydrous, analytical grade potassium nitrite, KNO_2 (dried at 105 °C for 1 hr) in distilled water. Add 1 ml 5 N NaOH and dilute to 250 ml. This solution contains 700 mg/l nitrite-N and should be stored in a dark bottle with 1 ml of chloroform as a preservative in refrigerator. The solution is stable for several months.

Procedure: Add 1.0 ml of sulfanilamide solution from a pipette to each 50 ml sample, mix and allow the reagent to react for more than 2 minute but less than 10 min. to assure a complete reaction. Add 1 ml of NED reagent and mix immediately. Leave for 10 minutes and then measure the absorbance (OD) of the samples and standards against a reagent blank at 540 nm. The colour is stable for 2 h. calculate the nitrite concentration by using calibration curve.

Nitrate-N

Principle: Nitrate in water sample is reduced almost quantitatively to nitrite. The nitrite produced is determined by diazotizing with sulfanilamide and coupling with NED to form a highly coloured azo dye which can be measured spectrophotometrically.

Reagents

- **Phenol solutions:** 23 g phenol in 500 ml of distilled water.
- **NaOH:** 1.25 g in 500 ml of distilled water.
- **Buffer reagent:** Mix equal volume of phenol solution and NaOH solution.
- **Copper sulphate solution:** 0.1 g in 1 litre distilled water.
- **Hydrazine sulphate:** 3.625 g in 500 ml of distilled water.
- **Reducing agent:** 5 ml of copper sulphate solution to 5 ml of hydrazine sulphate.
- **Acetone**
- **Sulfanilamide:** Dissolve 5.0 g in 50 ml of conc. HCl and make up the volume to 500 ml.
- **NED:** Dissolve 0.5 g of NED in 500 ml of distilled water
- **Nitrate standard solutions:** Dissolve 0.36119 g potassium nitrate, KNO_3 (AR dried at 105°C) in 250 ml distilled water. Dilute 100 ml of this solution to 1 litre with distilled water. This final solution contains 2 ppm NO_3^- .

Procedure

Take 10 ml of sample and add 0.4 ml of buffer and mix and then add 0.2 ml reducing agent and keep the tube in dark for 24 hours. Then add 0.4 ml of acetone and after 2 minutes add 0.2 ml of sulphanilamide. After 3 minutes, add 0.2 ml of NED solution and after 10 minutes, measure the absorbance at 540 nm in a spectrophotometer.

Floc volume Analysis: (Imhoff cone method)

It is a crude method that is the measure the floc volume by using Imhoff cone. One liter of water have to take and allow for settle half an hour. Measure the volume and note down. If higher the floc volume above 20ml/L reduce the feed level.



Turbidity

Principle: (Nephelometric method)

Turbidity can be caused either by planktonic organisms or by suspended soil particles. Turbidity due to suspended soil particles be measured by Nephelo-turbidity meter which is based on the scattering of light beam produced by tungsten filament lamp by particulate material. The quantity of light scattered is taken as a measured of turbidity in NTU. The higher the intensity of scattered light, higher the turbidity.

Reagents

- Turbidity freewater
- Standard turbidity suspension
- Solution-I: Dissolve 1g hydrazine sulphate in distilled water and dilute to 100 ml in a volumetric flask
- Solution-II: Dissolve 10g hexamethylenetetramine in distilled and dilute to 100ml.

Mix 5 ml each of Solutions I and II. Let stand 24 hours at 25°C . Dilute to mark and mix. The turbidity of this suspension is 400 NTU. Dilute 10 ml of this stock suspension to 40 NTU.

Procedure: Calibrate the instrument using standard turbidity suspension. Shake the sample thoroughly. Wait until air bubbles disappear and pour sample into turbidity meter tubes. Place the tube in instrument and read turbidity in NTU directly from instrumentscale.

Transparency

A standard Sacchi disc is a circular metal plate having 10 cm radius. The upper surface of the disc is divided into four quadrants, painted in black and white colours. The disc is gradually lowered into the water and depth (cm) at which the upper surface just disappears is noted (d1). Now the disc is slowly lifted upward and the depth at which the disc reappears is noted(d2). The value $(d1+d2)/2$ in cm gives a measure of transparency.

Periphyton biomass and its estimation

Periphyton biomass is estimated in terms of Dry matter (DM), ash free dry matter (AFDM) and chlorophyll a concentration.

Periphyton biomass

For estimation, take periphyton samples 2 x 2 cm² from submerged substrates in a pre weighed crucible and measure the wet weight of the sample. Dry the sample at 105°C for 24 h and keep it in desiccators. The difference between wet weight and dry weight will give the periphyton dry matter.

Ash free dry matter content in the periphyton dry can be estimated by keeping the samples at 450°C for 6 h in a muffle furnace and weigh the ash weight. The difference between the dry weight of the sample and weight of the ash gives the ash free dry matter (AFDM) content in the periphyton.

To determine chlorophyll concentration in periphyton biomass, transfer the periphyton samples to centrifuge tubes containing 90 % acetone and store overnight in a refrigerator. The samples were homogenize and centrifuged at 2,000 rpm for 10 min. Then measure the absorbance of the supernatant measure at 750 and 664 nm, 647 and 630 nm using a spectrophotometer. Estimate the Chllorphyll a content through tricromatic equations (APHA2005)

The autotrophic index (AI) of the periphyton biomass can be calculated using the following formula $AI = AFDM \text{ in } \mu\text{g cm}^{-2} / \text{Chlorophyll a in } \mu\text{gcm}^{-2}$.

2. MICROBIAL QUANTIFICATION AND CHARACTERIZATION IN BIOFLOC SYSTEM

Biofloc consists of non-living matter, and a variety of microorganisms, principally algae and bacteria. The presence of microorganism in biofloc confers many beneficial effects to aquatic animals. This includes provision of supplemental nutrition, maintenance of water quality and control of waste accumulation. Among microbial community, bacteria play crucial role in nitrogen cycle of pond ecosystem by converting toxic ammonia nitrogen into the bacterial single cell protein. Their optimum number is essential for the successful maintenance of biofloc system. On the other hand the very high number may lead to increased suspended solid, increased competition for oxygen with shrimp which may prove fatal at the time of crisis. Therefore, the estimation of microbial community is always at the centre-stage for management of biofloc system. The present chapter describes some common tools used in microbial estimation and characterization.

1. Total bacterial count

This roughly gives the estimate for total number of live and culturable bacteria exists in the system. These number should range from 10^6 to 10^8 CFU/mL.

Protocol

1.1 Media preparation

- Weigh 20g tryptone soya agar and dissolve in 500mL distilled water. This should be done in 1L conical flask.
- Add extra sodium chloride appropriately to bring the salt concentration of media to 2% for analysis of marine or brackish water samples. For the present experiment, add 7.5 g of sodium chloride in above media for our purpose.
- Autoclave the media at 121°C and 15 pond pressure for 15 min. This will kill all the germ and spore.
- Allow the media to cool.
- Pour 20ml media in 90 mm petridish plate inside laminar flow chamber. Keep the plate partially open for 30 minute to get the plate dried.
- Store the plate in refrigerator at 4°C.

1.2 Processing biofloc sample

- Collect 200 ml water sample from biofloc pond. Sample should be processed within 2-3h. Maintain the sample over ice or in refrigerator at 4°C.

- Homogenise the sample in kitchen blender for 30second. This will allow bacteria to get dissociated from thefloc.
- Make a serial 10 fold dilution of water sample in normal saline solution (0.89 g NaCl in 100 ml distilled water). Make 5 dilutions (10^{-1} to 10^{-5}) for normal pond and up to 8 dilutions (10^{-1} to 10^{-8}) for ponds having high load ofbiofloc.
- Spread plate 0.1 mL of diluted sample on tryptone soya agar plate. To prevent unnecessary wastage of plates, plating should be done starting from 10^{-3} dilution onward.
- Incubate the plate in inverted position for 24 h at 28 °C or at room temperature.
- Count the plate having 30 – 300 colony.

1.3 Calculation of bacterial count

Bacterial count is expressed in the form of colony forming unit (CFU). This is estimated by the following formulae.

Total CFU/mL or bacteria/mL = (no. of colonies x dilution factor) / sample volume used for plating

For example, suppose the plate of the 10^{-6} dilution yielded a count of 130 colonies. The sample volume used for plating is 0.1 mL. Then, the number of bacteria in 1 ml of the original sample can be calculated as follows:

$$\text{Bacteria/ml} = (130 \times 10^6)/0.1 = 1.3 \times 10^9$$

2.1 Total Vibrio count

Vibrio harveyi, *V. campbellii* and *V. parahaemolyticus* are the major bacterial pathogens in shrimp culture. Their predominance in biofloc system will signal danger; hence its monitoring should be taken as part of management criteria. Thiosulphate citrate bile salt sucrose (TCBS) agar is a selective cum differential medium widely used for *Vibrio* growth. It contains high concentrations of sodium thiosulfate and sodium citrate which inhibit the growth of Enterobacteriaceae. Growth of Gram- positive bacteria is inhibited by oxgall, a bile salt. It is a fermentable carbohydrate which helps in presumptive identification of *Vibrio* species. Forexample, *V. harveyi*, *V. campbellii* and *V. parahaemolyticus* are green while *V. alginolytiucs* and *V. cholerae* appear as yellow colony. There fore presence of very high number of green colony is considered as dangerous scenario in biofloc system.

Protocol

1. Add 8.9 g TCBS agar media for each 100 mL sterile distilled water.
2. The media should be boiled till the agar content gets completely dissolved. Please make a note that the TCBS agar should never be autoclaved.
3. Pour 20-25 ml media in Petridish, allow the plate to get dried.

4. After preparing the sample as earlier described method, spread plate 0.1 mL of diluted sample. Undiluted to 10^{-3} to 10^{-4} dilution can be plated on agar plate.
5. Count number of green and yellow colony.

4. MICROSCOPY IN BACTERIAL IDENTIFICATION

Bacterial cells are very small in size, usually in the range of $0.5\ \mu$ to $20\ \mu$. Limit of vision of human eye is just 100μ , so they are not visible to naked eye. Hence a microscope is used for observing bacterial cell. They are semi-transparent, so, we use suitable dyes to stain them to obtain better contrast. Gram staining is the most common staining procedure followed in microbiology lab as a first step aid in bacterial identification.

4.1 Grams staining

It was designed by Christian Gram in 1884. The primary staining is done by crystal violet and counter staining by Safranine.

Procedure

1. Smearpreparation: Young bacterial cultures of 16-24h shall be used for staining. Take dust free and oil free microscopic glass slide. Take a speck of young culture with the help of inoculation loop and emulsify with a drop of sterile water in the middle of the slide and spread uniformly. Dry the smear in the air.
2. Smearfixation: Fix the smear by passing the slide 3-4 times quickly through the blue flame of Bunsen burner, with the smeared side on the top. Take precaution to not char the smear.

3. Staining

- Primary staing: Flood the smear with Gram's crystal violet - 1 min.
- Wash with distill water drop by drop.
- Moderant: Flood the smear with Gram's iodine - 1 min.
- Wash with water drop by drop.
- Destaining: Destain with drop-wise addition of ethyl alcohol until washings are free from violet color
- Wash with water.
- Counter staining: Counter stain with Safranine - 1 min.
- Wash with water.
- Dry in air.

4. Microscopy

Observe the slides under the microscope using oil immersion objective (100X). Note down following points

- Gram positive or Gram negative - cells stained violet, bluish violet or purple are Gram positive. Bacteria appearing pink are Gram negative.
- Shape, size and arrangement of cells, i.e., Cocci, Bacilli, Spiral or curved rod, Filamentous etc. For cocci whether they are in single or pairs or chains or bunch.
- Examine whether the culture is pure or mixed.
- If the cells are Gram-positive rods, examine whether there is spore formation. If spore is present, there will be unstained area inside the cell, because, spore is not stained by Grams method.

4.2 Bacterial Identification by biochemical methods

Biochemical methods are routinely followed in microbiology lab for bacterial identification. Though the test is laborious, it is cheaper and requires less input. Therefore, it could be easily practiced even at remote area with primary level facility like incubator, laminarflow and autoclave. We are providing the list of biochemical tests routinely done in our lab for identification of *Vibrio* species with description of few commonly used tests.

5. Biochemical tests used for *Vibrio* identification

- Carbohydrate fermentation test–Sucrose, arabinose, lactose, Mannose, ONPG
- Salt tolerance test–Growth of *Vibrio* species at 0, 3, 6, 8 and 10% salt in peptone water
- Decarboxylase test – Lysine, arginine, ornithine
- IMViC test–This is a acronym for Indole, Methylred, Voges Proskauer and citrate test.

5.1.1 Carbohydrate fermentation test

Materials

1. Phenol red broth base containing phenol red indicator.
2. Carbohydrate solution – Add 10 gm of carbohydrate in 20.0 ml of distil water. Prepare different carbohydrate solution such as Glucose, Sucrose, arabinose, lactose, Mannose, ONPG etc solution separately in a properly labelled flask.
3. *Vibrio* culture

Procedure

1. Sterilise phenol red broth base at 15 pound pressure for 15 minute and carbohydrate solution at 10 pound pressure for 10min. Separately label each carbohydrate solution. Add 0.2 ml of carbohydrate solution in each test tube containing 9.8 ml of phenol red broth.
2. Using aseptic technique inoculate each tube with bacterial culture.
3. Place the tubes in a test-tube rack and incubate at 30°C for 24 to 48 hours.
4. Examine the tubes carefully at 4 hours, 8 hours, and 18 hours for evidence of acid (A), or acid and gas (A/G) production. Acid production is detected by the medium turning yellow and gas production by a gas bubble in the Durham tube. Purple color indicate no fermentation.

5.1.2. Salt tolerance test Procedure

1. Prepare peptone water having 0, 3, 6, 8 and 10% salt. Autoclave at 15 pound pressure for 15min.
2. Transfer these media into sterile test tube and inoculate with a drop of culture.
3. Incubate at 28 °C for up to 4 days and check for presence of growth daily.
4. Presence of turbidity in the medium should be considered as positive.

5.1.3. Decarboxylase test

Lysine, Ornithine, and arginine are the three amino acids routinely tested for decarboxylase test.

Procedure

1. Prepare Moeller decarboxylase base with lysine, ornithine and arginine hydrochloride (1%) in a separate test tube.
2. Inoculate the test medium with overnight grown inoculum and overlaid with liquid paraffin layer.
3. Incubate the tube at 28°C and read daily for four days.

Result

- Purple color-Positive Decarboxylation
- Yellow color or no color change -Negative i.e. No decarboxylation

5.1.4 IMViC test

5.1.4.1 Indole test

Indole test indicates the presence of tryptophanase enzyme which hydrolyse tryptophan to its metabolic products, namely, indole, pyruvic acid, and ammonia. The presence of indole is detected by the addition of Kovacs' reagent, which after reacting with indole produces a bright red compound on the surface of the medium.

Procedure

1. Prepare tryptic soy broth tube.
2. Using aseptic technique, inoculate each tube by a loop full of culture.
3. Incubate the tubes for about 24 hours at 28°C.
4. After 24 hours, add 0.5 ml (about 10 drops) of Kovacs' reagent to each tube, and shake the tube gently. A deep red colour develops in the presence of indole. Negative reactions remain colorless or light yellow.

Methyl red and Voges Proskauer test the end product coming from glucose metabolism. While citrate tests identify the utilization of citrate as sole carbon source by bacteria.

6. Bacterial identification by 16S rRNA gene sequencing

The 16SrRNA gene sequence is the most widely used molecular method for bacterial identification and phylogenetic study. There are multiple reasons for selection of 16Sr-RNA genes which include

- a) It is present in almost all bacteria
- b) The function of the 16SrRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution).

6.1. Isolation of genomic DNA

1. Take 1.5 ml of a 6-8 h old *Vibrio* culture. Harvest bacterial cells at 4000 x g for 5min.
2. Suspend the cell pellet in 400µl of lysis buffer (40mM Tris-acetate pH7.8, 20mM sodium-acetate, 1 mM EDTA, 1% SDS) by vigorous pipetting.
3. Incubate at 65 °C for 20min.
4. Add 3 micro liter of RNase. Mix by inverting the tube. Incubate for 30 min at 37°C.
5. To remove most proteins and cell debris, add 132µl of 5M NaCl solution. Mix well by inversion.
6. Centrifuge the viscous mixture at 12,000 rpm for 10 min at 4°C.
7. Transfer the clear supernatant into a new vial. Add an equal volume of chloroform. Invert the tube at least 50 times when a milky solution completely formed.
8. Centrifuge at 12,000 rpm for 5 min, transfer the supernatant to another vial.
9. Precipitate DNA with equal volume of 100% ethanol. Invert few times to get the visible pellet.

DNA

1. Washed twice with 70% EtOH.
2. Dry the tube in air for 10-20min.
3. Redissolve isolated DNA in 100 μ l 1 x TE buffer.
4. Keep at 4°C for overnight to get the pellet dissolved otherwise heat at 65 oC for 10 min todissolve the DNA pellet.

6.2. Preparing PCR reaction solution

Prepare a master mix by mixing PCR component as following

Component	25 μ l reaction	Final Concentration
10X Standard <i>Taq</i> Reaction Buffer	2.5 μ l	1X
10 mM dNTPs	0.5 μ l	200 μ M
10 μ M Forward Primer	0.5 μ l	0.2 μ M (0.05–1 μ M)
10 μ M Reverse Primer	0.5 μ l	0.2 μ M (0.05–1 μ M)
Template DNA	1 μ l	10-100 ng
<i>Taq</i> DNA Polymerase	0.125 – 0.5 μ l	0.5 to 1 units/25 μ l PCR
Nuclease-free water	to 25 μ l	

Notes: Gently mix the reaction. Collect all liquid to the bottom of the tube by a quick spin

6.3. PCR reaction setup

Thermo cycling conditions for a routine PCR:	Temp	Time
Initial Denaturation	95°C	4 min
30 Cycles	95°C	30seconds
	57°C	45seconds
	72°C	1 min 30 second
Final Extension	72°C	7 minutes
Hold	22°C	

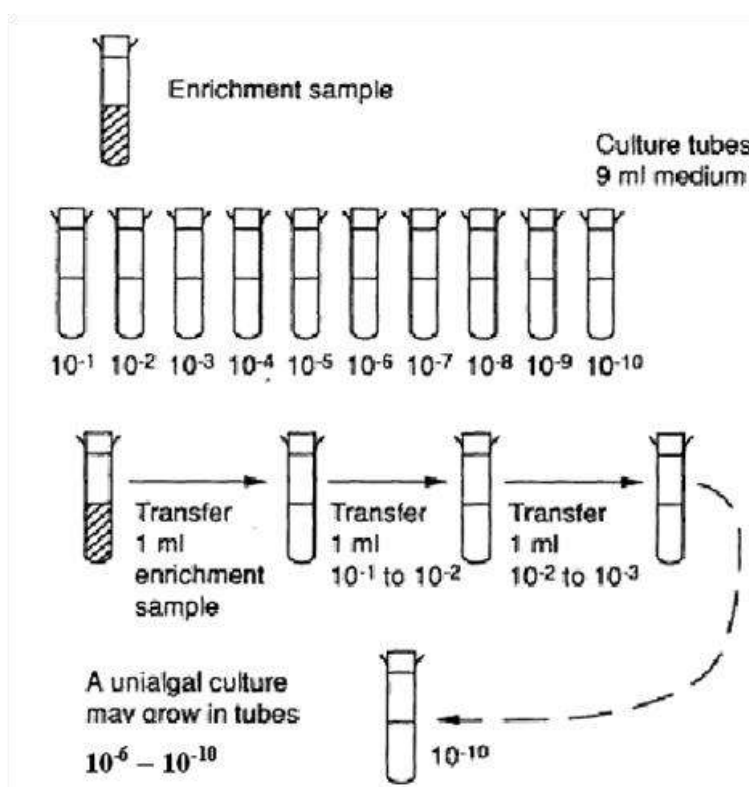
Run the PCR products by agarose gel electrophoresis at 1.2% concentration at 100 V for 1 h. Visualize the PCR product under UV ray or gel documentation system. The PCR product should be sequenced and examined for homology by BLAST analysis.

5. ALGAL CHARACTERIZATION

Out of several methods of microalgal isolation; three important methods are explained here: serial dilution, agar streak plating and manipulation.

1. Serial dilution

Using aseptic technique, dispense 9 ml of specified chemical media (eg f/2 medium) into each of ten test tubes with sterile automatic dispenser or sterile 10ml pipettes. Label tubes 10^{-1} to 10^{-10} indicating the dilution factor. Aseptically add 1 ml of enriched/fresh sample to the first tube (10^{-1}) and mix gently. Take 1 ml of this dilution and add to the next tube (10^{-2}), mix gently. Repeat this procedure for the remaining tubes (10^{-3} to 10^{-10}). Incubate test-tubes under controlled temperature and light conditions. Temperature and photoperiod should be as close to the natural environment as possible. Light intensity should be slightly lower than the natural environment.

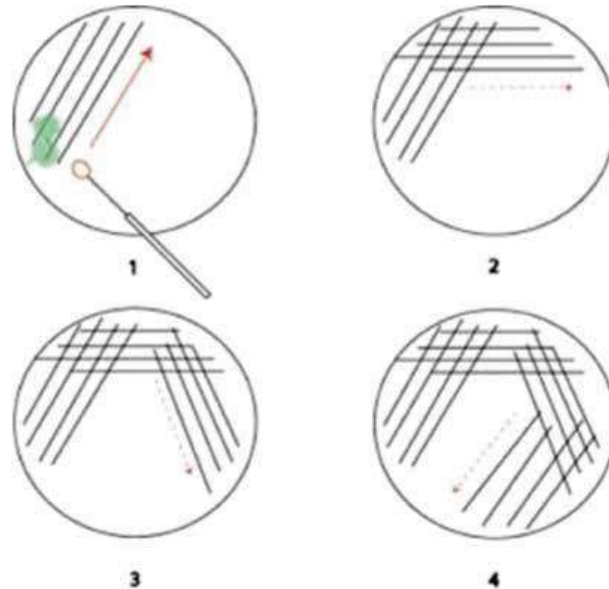


Then examine cultures microscopically after 2-4 weeks by withdrawing a small sample aseptically from each dilution tube. A unialgal culture may grow in one of the higher dilution tubes e.g. 10^{-6} to 10^{-10} . If tubes contain two or three different species methods like micromanipulation or agar plate streaking can be used to obtain unialgal cultures.

Fig 1: Test tubes in serial dilution

2. Agar streak plating

This is a suitable method for small species or algae that grow well on a substrate. Prepare petridishes containing growth medium solidified with 1-1.5% agar. The agar should be 1/2-2/3 the depth of the dish. Place 1-2 drops of mixed phytoplankton sample near the periphery of the agar. Sterilize wireloop in flame. Use the sterile loop to make parallel streaks of the suspension on the agar. Note that there are 16 streaks (4 sets of 4) to be made and the whole surface of the agar plate



is used. Cover and seal plate with parafilm. Invert and incubate under low light at constant temperature. Select colonies that are free of other organisms for further isolation. Remove a sample using a sterilized wire loop and place in a drop of sterile culture medium on a glass slide. Check microscopically that the desired species has been isolated and is unialgal. Repeat the streaking procedure with the algal cells from a single colony and again allow colonies to develop. This second streaking reduces the possibility of bacterial contamination and of colonies containing more than one algal species. Transfer selected colonies to liquid or agar medium.

3. Micromanipulation

Micromanipulation is a powerful tool in microalgal isolation. It is usually performed with a pasteur pipette or a glass capillary. A Pasteur pipette can be heated in a flame, extended, and broken. With minimal practice, this technique becomes quick and easy, but the beginner must spend some time practicing before reliable production of micropipettes is achieved. The pipette is held in one hand, and a forceps held in the other hand supports the tip. The pipette is rotated to provide even softening as the pipette warms to the melting point. When the heated area is sufficiently soft, the pipette is removed from the flame and simultaneously pulled to produce a thin tube. If drawn out too quickly, or if drawn out in the flame, then the thin extension breaks or burns through, and the resulting product is unsatisfactory. The goal of micropipette isolation is to pick up a cell from the sample, deposit the cell without damage into a sterile droplet, pick up the cell again, and transfer it to a second sterile droplet. This process is repeated until a single algal cell,

free of all other protists, can be confidently placed into culture medium. The process balances two factors: cell damage by excessive handling, which is bad, and clean isolation of a single cell, which is good. For robust organisms, repeated handling can be achieved without damage; however, for delicate organisms, cell damage is an important concern.

6. IMMUNE PARAMETERS CHARACTERIZATION

The immune response in invertebrates is innate rather than adaptive and has no known immunological memory. Immunity in shrimps is defined as nonspecific internal defense response that includes humoral, cellular and molecular components.

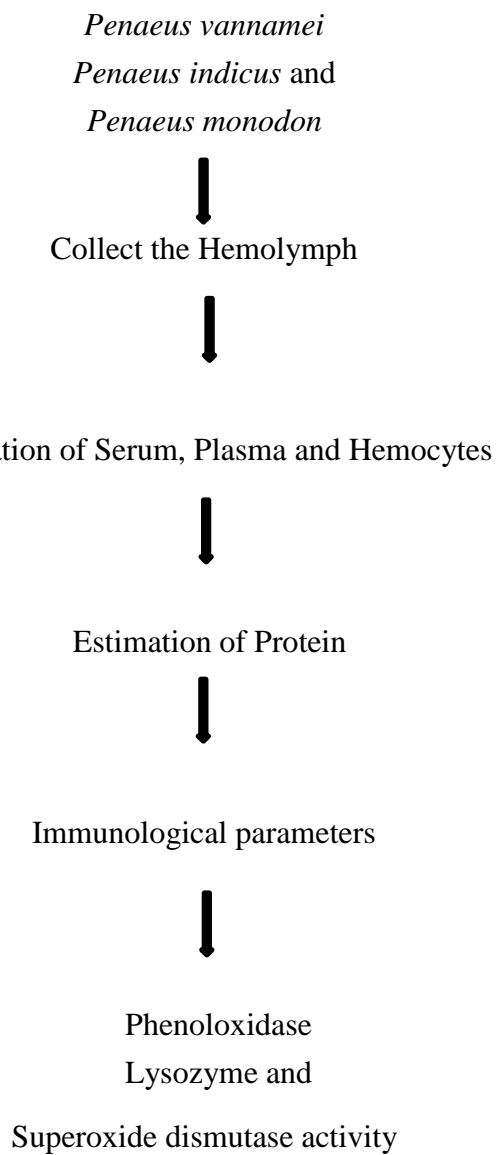
Humoral: immune response involve plasma proteins and peptides with different actions, including recognition proteins, clotting protein, anti-microbial peptides, agglutinin, lectin and the proPO system

Cellular: immune system constituted by phagocytic process,encapsulation, nodule formation, ProPO activating system, clotting system and reactive oxygen intermediates (ROIs) created by the metabolites from respiratory process. Different kinds of haemocytes: granular, semi-granular, and hyalins, are involved in activating these functions (adhesion, phagocytosis, encapsulation, and melanization).

Molecular: immune response constituted by the immune genes involved for various immune reactions and their qualitative and quantitative assessments. For every cellular and humoral defense, one or many complex interactions of genes are involved, some are known and majorities are yet to be sequenced and characterized.

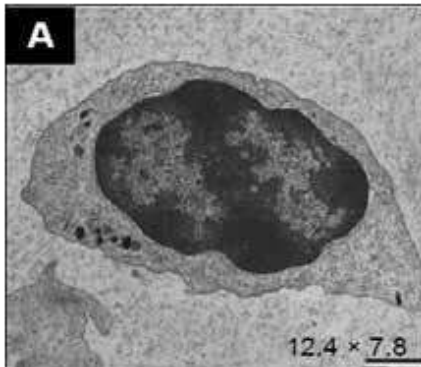
Bacteria and yeast have molecules such as lipopolysaccharide (LPS) and β -1, 3-glucans on their surfaces that are recognized by non self recognition molecules and elicit an immune reaction in both vertebrates and invertebrates. Proteins that recognize the LPS and β -1, 3-glucans are known as pattern recognition proteins (PRPs) and differentially regulated in normal and diseased animal.

SCHEMATIC DIAGRAM OF IMMUNOLOGICAL PARAMETERS

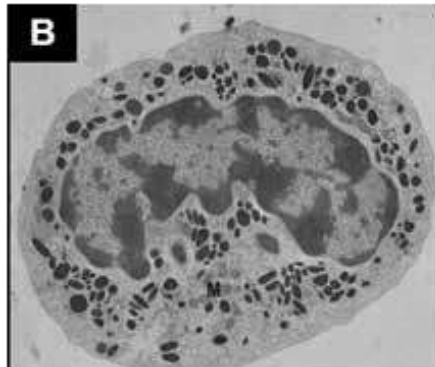


SCHEMATIC DIAGRAM OF PHAGOCYTOSIS IN CRUSTACEANS HEMO-CYTES

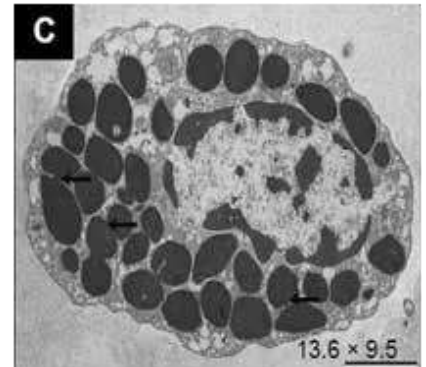
Hyalinehemocyte



Semigranularhemocyte



Granularhemocyte



Semi granular or Granular hemocyte



Adherence of microbe or foreign particle to phagocyte



Ingestion of microbe or foreign particle by phagocyte



Formation of a phagosome



Fusion of the phagosome with a lysosome to form a phagolysosome



Digestion of ingested microbe or foreign particle by enzymes



Formation of residual body containing indigestible material



Discharge of waste materials

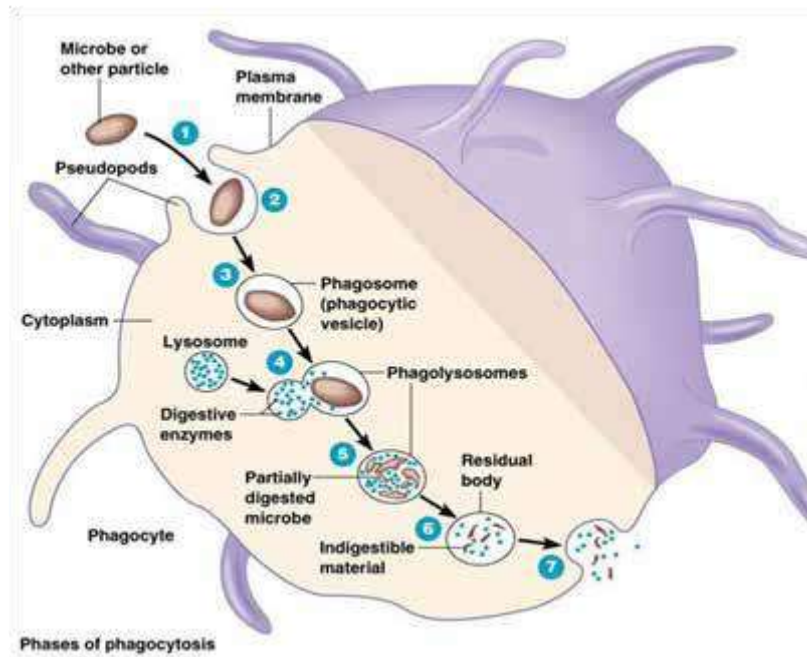


Fig.1. Phagocytosis of hemocyte

PHENOLOXIDASE ACTIVITY SCHEMATIC METHODOLOGY

Penaeus indicus, *Penaeus vannamei* and *Penaeus monodon*



Take the 100 μ l of sample



Added 100 μ l of Trypsin (5 mg/ml)



Added 1.8 ml of 5 mM L-DOPA (Tris-HCl 50 mM, pH 7.5 buffer)



Incubation for 20 minutes



OD at 490 nm

PHAGOCYTOSIS SCHEMATIC METHODOLOGY

Hemolymph (100 μ l) collected in 1 ml of tri sodium citrate buffer (30 mM tri sodium citrate, 340 mM NaCl, 10 mM EDTA, 120 mM dextrose; pH 7.55)



Spread on a clean, dry glass slide over an area of 2 cm²

Kept in a moist chamber for 30 min at 23°C



To obtain hemocyte monolayer (50 μ l)

Monolayers was overlaid with 50 μ l of bacteria



Observed at 20 or 30 min after under light microscope at 40x magnification

TOTAL HAEMOCYTE COUNT SCHEMATIC METHODOLOGY

Hemolymph (100 μ l) collected in 1 ml of tri sodium citrate buffer (30 mM tri sodium citrate, 340 mM NaCl, 10 mM EDTA, 120 mM dextrose; pH 7.55)



After they were mixed gently hemocytes



Added 10 μ l of hemocytes in hemocytometer



Under light microscope at 40x magnification



Count, calculated as cells ml^{-1}

LABORATORY METHOD FOR DETECTION OF IMPORTANT SHRIMP PATHOGENS FROM BIOFLOC CULTURE SYSTEM

Shrimp aquaculture is a highly profitable industry which has been constantly developing through the application of scientific knowledge and developed technologies. Through this process, it has been possible to increase the production to several folds by intensification of culture process. Biofloc technology is one of the similar methods where a very high stocking density shrimp culture is possible through the exploitation of the natural ecosystem. However, disease always comes on the way to success of any aquaculture practice. Any alteration to the normal conditions can bring stress on the organism during which the animals become susceptible to various pathogens. It is also possible that entry of pathogens can occur through various sources. Therefore, constant monitoring of culture practices is essential. Following are the protocols for detection of some important pathogens in Indian shrimp aquaculture practices. These methods can be routinely adopted for the monitoring of the biofloc culture practice for the early detection of pathogens and then take necessary actions either for treatment or to prevent the spread.

White Spot Disease (WSD)

This is considered as a serious disease as it can bring mass mortality once the culture system gets contaminated. Mortality starts after 2 to 3 days onset of disease and mass mortality may occur within 7-10 days. This disease struck Indian shrimp aquaculture during late 90's and even after two decades of occurrence, it still poses threat to the industry. All the important cultured penaeid shrimps and all their life stages can get infected by the virus. White Spot Syndrome Virus (WSSV) is the causative agent, which is a double stranded circular DNA virus of about 300 kb. This pathogen has large number of crustaceans as carrier and hence once contaminated, it becomes difficult to eliminate.

Clinical signs

Different clinical symptoms include anorexia and coming to surface or pond sides. Shrimps become lethargic and during this time the chance of disease spread increases through cannibalism. Animals may show pink discoloration. One of the characteristic clinical sign includes development of circular white spots on the carapace and entire abdomen. However, in some cases, particularly



in case of vannamei, white spots may not be visible. Feeding is considerably reduced or completely stopped.

Disease diagnosis

There are various methods such as direct microscopy, histopathology, in situ hybridization, polymerase chain reaction (PCR), real time PCR or loop mediated isothermal amplification (LAMP) are available for the detection of WSSV.



However, amongst all, PCR is widely used as it is rapid, sensitive and easy to perform compared to other methods.

Different steps in PCR diagnosis of WSSV

Sample collection: Live, moribund, freshly dead and frozen samples are suitable for PCR detection. Samples can be processed directly or stored in 90-100% ethanol for future analysis. While cuticular epithelium and sub-cuticular connective tissues are considered as best samples for WSSV, hepatopancreas, midgut and compound eye are regarded as bad samples. As non-lethal sampling protocol, tips of pleopods can be cut and used.

DNA extraction: About 100 – 200 mg tissues or 100 µl of haemolymph is sufficient for DNA extraction. Basic principle involves 4 steps – Tissue lysis, removal of inhibitors (protein, lipid etc), precipitation of DNA and washing to remove extra salt. Several methods of DNA extraction protocols are available. OIE manual describes CTAB method. Similarly, Guanidine Hydrochloride can also be used. Some of the commercially available kits mention partial purification protocols to have the crude DNA for detection. To get the accurate result, it is advisable to use some of the established methods.

Thermo cycling: Different components such as PCR buffer, pair of primers, dNTPS and DNA polymerase enzyme are necessary to set up a PCR reaction. Readily available master mix containing buffer, dNTPs and polymerase enzymes is also available. Depending on the number of reactions, a master mix is prepared. All the PCR components except the DNA should be handled in a PCR work station and in cold condition to avoid contamination. A typical PCR reaction can be of 20, 25, 50 or 100 µl. Nested PCR protocols are available for WSSV detection

Example

2X master mix:

12.5 µl Forward Primer (10pm):

0.5 µl

Reverse Primer (10pm): 0.5µl

DNA: 1.0 µl (or depending on concentration)
PCR grade water: 10.5 µl

Total: 25µl

Different primers available in the research publications may be used. In case of commercial kits, the primers are already added to the master mix.

With each PCR sample, a positive control (known WSSV positive sample) and a negative control (known WSSV negative shrimp) should be there. It is also advisable to keep one reaction for one of the suitable host DNA to check the quality of DNA extraction.

The components are mixed properly and centrifuged briefly. This is then loaded to a thermocycler. Thermo cycling involves one initial denaturation step (usually 94 °C) for about 5 minutes, followed by repeated cycles (30 – 40 numbers) consisting of denaturation (usually 94 °C for 30 sec to 1 min), annealing (depends on T_m of primers, 30 sec to 1 minutes) and extension (usually 72 °C, 30 sec to 2 minutes). A final extension (72 °C) for about 5 -10 minutes is given to complete their action.

A typical example for WSSV by OIE protocol: 94°C for 4 minutes, 55°C for 1 minute, and 72°C for 2 minutes, followed by 39 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes and a final 5-minute extension at 72°C.

Detection

The PCR amplified product is detected in an agarose gel (1-2% depending on size of the amplified product). Either Tris Acetic acid and EDTA (TAE, 1X concentration) or Tris Boric acid EDTA (TBE, 0.5 X concentrations) buffer is used for electrophoresis. Ethidium bromide (0.5 µg/ml) can be incorporated to the gel (care should be taken not to touch EB as it is carcinogenic). The amplified products are loaded to the wells of agarose gel and then connected to a power pack (initial 80V and increased to 120 or 140 V once the products enter the gel).

The agarose gel is then visualised in a Gel documentation system or UV-transilluminator. A molecular weight marker is put to verify the size of the amplified products.

Infectious Hypodermal Haematopoietic Necrosis Virus (IHHNV)

Though this virus does not bring mortality, it has been reported to cause body deformity (Rund deformity syndrome or RDS) and growth variation. Farmers become more worried as it brings size variation and growth reduction. Both WSSV and IHHNV are frequently being reported from Indian shrimp aquaculture system.

Clinical signs

Shrimps affected by IHHNV show RDS, smaller size compared to control and there will be large scale size variation. Usually mortality is not recorded due to IHHNV infection. However, under severe stress conditions, these shrimps may show mortality.

Disease diagnosis

Similar to WSSV, IHHNV can also be detected by histopathology, PCR, Realtime PCR, *in situ* hybridization and LAMP. Here also, PCR is considered as most popular.

Different steps in PCR diagnosis of IHHNV

IHHNV has similar targets as WSSV. Therefore, all the samples used for WSSV detection, can also be used for IHHNV detection. DNA extracted for WSSV detection can also be used for IHHNV detection. Different primers have been used for detection of IHHNV. Care should be taken to avoid primers that can detect integrated IHHNV to shrimp genome. Several commercially available kits are also available. Nested PCR protocols are available for IHHNV detection. Amplification of products and agarose gel detection will be similar to that of WSSV.

Enterocytozoonhepatopenaei (EHP)

This is also called as hepatopancreatic microsporidiosis (HPM) and is an emerging disease that affects seriously to many of the farms in South East Asian countries and in India. First reported as an un named micro sporidian from growth retarded giant or black tiger shrimp *Penaeus monodon* from Thailand and was subsequently characterized in detail. It also has much smaller spores (approximately 1µm in length) and is currently known to infect both *P. monodon* and *P. vannamei*. It has been found that EHP can be transmitted directly from shrimp to shrimp by cannibalism and cohabitation

Clinical signs

There are no specific clinical signs for EHP. Associated slow growth and size variations are the only clinical signs. No mortality has been reported to be associated with EHP. Shrimps affected with white gut syndrome are of ten found to be positive for EHP but a strong correlation has not yet been confirmed.

Disease detection

Presently direct microscopy, histopathology, in situ hybridization, PCR, Realtime PCR and LAMP is being used for the detection of EHP.



Different steps in PCR diagnosis of

Sampling: The target organ for EHP is the hepatopancreas. The spores also come out through the intestine in the faecal strands. Therefore, as a non-lethal sample protocol, the faecal strings can also be used for EHP detection.

DNA extraction: The principle of DNA extraction is similar to that of WSSV and IHHNV. As the target organs here are expected to contain more PCR inhibitors, it is suggested to use one established method to make sure that the DNA obtained is pure enough to avoid any false positive.

Thermocycling: Basic principles are similar as above. Here the primers sequences are different. Accordingly, the cycling conditions also vary. A nested PCR protocol is available for EHP detection.

Detection

The amplified product is detected in similar way as described above.

Primer Used for Disease Screening

Disease			Forward	Rev	Base pairs
WSSV	OIE	First	5'-ACT-ACT-AAC-TTC-AGC-CTA-TCTAG-3	5'-TAA-TGC-GGG-TGT-AAT-GTT-CTT-ACG-A-3'	1447
		Nested	5'-GTA-ACT-GCC-CCT-TCC-ATC-TCC-A-3'	5'-TAC-GGC-AGC-TGC-TGC-ACC-TTG-T-3'	941
	Kimura primers	First	5'ATCATGGCTGCTTCACAGAC-3'	5'-GGCTGGAGAGG ACAAGACAT-3'	982
		Nested	5'-TCTTCATCAGATGCTACTGC-3'	5'-TAACGCTATCCAGTATCACG-3'	570
IHHNV			5-GGG-CGA-ACC-AGA-ATC-ACT-TA- 3	5'-ATC-CGG-AGG-AAT-CTG-ATG-TG- 3	389
EHP	First	5'-CAGCAGGCGCGAAAATTGTCCA-3'	5'-AAGAGATATTGTATTGCGCTTGTG-3	779	
	Nested	5'-CAACGCGGGAAAACTTACCA-3'	5'-ACCTGTTATTGCCTTCTCCCTCC-3'	176	

RNA EXTRACTION PROTOCOL

Homogenize 50-100mg tissue/ 10^6 cells in 1ml TRI soln. Centrifuge @ 12,000 rpm for 10 mins @ 4°C.



Add 0.2ml chloroform per 1 ml TRI soln for phase separation, Centrifuge @ 12,000 rpm for 15 mins @ 4°C.



To the supernatant add 0.5ml isopropanol and precipitate RNA by incubating at RT for 10 mins. Centrifuge @ 10,000 rpm for 15 mins @ 4°C.



Wash RNA pellet with 1ml of 75% Ethanol per 1ml of TRI soln used. Centrifuge @ 12,000 rpm for 15 mins @ 4°C.



Air dry the pellet. Resuspend RNA pellet 35-100µl RNase free water.

Quantification of RNA: 50ul cuvette: 10M Tris-cl DEPC water
49ul of sterile or DEPC water+ 1ul of RNA



Vortex for few seconds



The 5ul of sample is quantified in biophotometer 540 nm.

Purity of RNA should be around 1.8 to 2.0 at 260/280 value

Calculation

=40 x 260 OD value X 50

=value X 0.050

=Value/50ul

=convert the value for 1ul concentration.

Synthesis of cDNA

About 2µg concentration of total RNA solution was taken. A volume of 4µl Dntp 2.5mM mix was added. Final volume was made to 16µl with nuclease free water. The sample was allowed to heat for 3-5 mins at 65-70°C. Allowed to spin briefly and placed on ice. A volume of 2µl 5X RT buffer, 1µl of RNase inhibitor, and 1µl of MuLV Reverse Transcriptase enzyme were added. Incubated at 42°C for 1hr. The enzyme was inactivated at 80°C for 5mins. Stored at -20°C for further analysis.

PCR Cycles

Initial denaturation: 95°C (3 min)

Denaturation : 95°C (30 sec)

Annealing temperature (based on primer): 30 sec

Extension : 72°C (30 sec)

Final Extension : 72°C (5 min)