EFFECT OF BIOFLOC ON THE PRODUCTION OF *LITOPENAEUS VANNAMEI* (BOONE, 1931) AND CHARACTERIZATION OF MICROBIAL COMMUNITY IN BIOFLOC

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by

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Dated: 26th June 2013

CERTIFICATE

Certified that the dissertation entitled **"EFFECT OF BIOFLOC ON THE PRODUCTION OF *LITOPENAEUS VANNAMEI* (BOONE, 1931) AND CHARACTERIZATION OF MICROBIAL COMMUNITY IN BIOFLOC"** is a record of independent bonafide research work carried out by **Mr. M. RAJKUMAR** during the period of study from September 2012 to June 2013 under our supervision and guidance for the degree of **Master of Fisheries Science (Aquatic Environmental Management)** and that the dissertation has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title.

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DECLARATION

I hereby declare that the dissertation entitled "**EFFECT OF BIOFLOC ON THE PRODUCTION OF *LITOPENAEUS VANNAMEI* (BOONE, 1931) AND CHARACTERIZATION OF MICROBIAL COMMUNITY IN BIOFLOC**" is an authentic record of the work done by me and that no part thereof has been presented for the award of any degree, diploma, associateship, fellowship or any other similar title.

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ABSTRACT

The effect of biofloc on the water quality and growth performance of *Litopenaeus vannamei* (Boone, 1931) was conducted by culturing the animals for 90 days in indoor tanks of 500 L capacity. Three biofloc treatments i.e. sugarcane molasses (BFT_S), tapioca flour (BFT_T), wheat flour (BFT_W) and a control in triplicate were maintained for 30 days before starting the culture experimet. The PL with an average body weight of 0.15 ± 0.02 g were stocked at the rate of 130 PL m^{-2} and feed was applied @ 1.5% of the total biomass. The total suspended solid (TSS) level was maintained at around 500 mg L^{-1} in all the treatments. The addition of carbohydrate significantly ($P<0.05$) reduced the total ammonia-N (TAN), nitrite-N and nitrate-N and also increased the total heterotrophic bacterial (THB) population in the treatments. There was a significant differences ($P<0.05$) between the treatments and control groups in terms of final average body weight (ABW), food conversion ratio (FCR) and growth rate. The BFT_W showed significantly higher growth rate (0.09±0.001 g day^{-1}) and ABW (8.49±0.09 g) than the other treatments and control. Survival of the shrimps was not affected ($P>0.05$) by the treatments and it ranged between 82.2% and 90.3%. The nutritional quality of the biofloc and the proximate composition of the shrimp were significantly different between the treatments and control. Tintinids, ciliates, copepods, cyanobacteria and nematodes were observed in all the treatment tanks. The bacterial community profile of bioflocs was analysed by denaturing gradient gel electrophoresis (DGGE) by amplifying bacterial 16S rRNA gene. From sequence analyses, *Ruegeria* sp. *Chitinophaga pinensis, Roseobacter litoralis, Hyphomonas neptunium* and *Phaeobacter gallaeciensis* were found to be the most dominant bacteria in all the treatments. It can be concluded that the use of wheat flour (BFT_W) effectively enhances the biofloc production which contributed effectively in maintenance of good water quality resulting higher production of shrimp.

सारांश

९० दिनों के लिए ५०० ली. टैंक में बायोफ्लॉक का प्रभाव जल के गुणवत्ता तथा *लिट्रोपेनिअस बन्नामेयी* (बून, १९३१) में वृध्दि अनुपालन को जानने के लिए अध्ययन किया गया। इस अध्ययन में उत्पादन प्रयोग के ३० दिन पहले बायोफ्लॉक अभिक्रिया के लिए गन्ने की चाशनी (BFT_S), टैपिओका (BFT_T), गेहूँ का आटा (BFT_W)) तथा नियंत्रण को त्रिगुणित करके लिया गया। १३० PL/ मी.^{2} की दर से ०.१५ $\pm$ ०.०२ ग्रा. का औसतन शारीरिक वजन वाला PL लिया गया / चारा का प्रयोग कुल जैवमात्रा के १.५% के दर से किया गया। कुल निलंबित ठोस का स्तर सभी अभिक्रियाओं में लगभग ५०० मि.ग्रा./ली. रखा गया। कार्बोहाइड्रेट को मिलाने से कुल अमोनिया-नाइट्रोजन, नाइट्राइट-नाइट्रोजन तथा नाइट्रेट-नाइट्रोजन में महत्वपूर्ण रूप से ($P<0.05$) कमी हुई। औसतन शारीरिक वजन, आहार परिवर्तन अनुपात तथा वृध्दि दर में एक महत्वपूर्ण अंतर पाया गया। दुसरे अभिक्रियाओं तथा नियंत्रण के तुलना में BFT_W ने सार्थक रूप से अधिक वृध्दि दर (०.०९$\pm$०.००१ ग्रा./दिन) तथा अंत में औसतन शारिरीक वजन (८.४९$\pm$०.०९ ग्रा.) दिखाया। दुसरे अभिक्रियाओं तथा नियंत्रण की तुलना में अधिक आहार परिवर्तन अनुपात BFT_W (९.०३$\pm$०.०३) में पाया गया। झींगा के उत्तरजिविता में सार्थक रूप से ($P<0.05$) कोई प्रभाव किसी अभिक्रिया में नहीं पड़ा तथा इसका मान ९२.२% और ९३.३% के बीच था। बायोफ्लॉक तथा झींगा के पोषक गुणवत्ता में सार्थक रूप से अभिक्रिया तथा नियंत्रक में अंतर पाया गया। सभी अभिक्रियाओं में टिंटिनीड्स, सिलिएट्स, कोपीपोड, सायनोबैक्टिरिया तथा नीमेटोड्स पाया गया। बायोफ्लॉक में
जीवाणुओं के समुदाय के रूपरेखा का विश्लेषण विकृत अनुपात जैल इलेक्ट्रोफोरेसिस (DGGE) के द्वारा विस्तारित जीवाणुओं के १६S रायबोसोमल RNA की सहायता से किया गया। सभी अभिक्रियायों के विश्लेषण के बाद *रूगेरिया जाति*, *कीटोनोफेगा पिनेनसिस*, *रोसियोबैक्टर लीटोरेलिस*, *हाइफोनोनस नेप्टुनियम तथा फियोबैक्टर गेलेसिएंसिटा* जीवाणु सबसे प्रबल पाया गया। यह कहा जा सकता है कि गेहूँ के आटे का उपयोग सार्थक रूप से बायोफ्लॉक के उत्पादन को बढ़ाता है जो अच्छे जल के अनुरक्षण के द्वारा अधिक झींगा उत्पादन में मदद करता है।

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF PLATES

1. INTRODUCTION

World aquaculture production has grown by approximately 8.3% per year from 1970 to 2008, reaching to the tune of 52.5 million tons. This represents 36.9% of the total worldwide captured fish production (FAO, 2010). In aquaculture, crustaceans have good demand and economic value both in domestic and international markets. According to the Food and Agriculture Organization of the United Nations (FAO, 2003; 2008*)*, shrimp is the most important and profitable commodity among all seafood trades. Shrimp culture has a significant exploration among farmers, contributing to world's aquaculture production, but concerns about the environmental impacts due to this activity have also increased (Tovar *et al.,* 2000; Jory *et al*., 2001). The environmental impacts of aquaculture practices are identified as effluent discharging and the eutrophication of adjacent water bodies, the introduction of exotic species and outbreak of diseases (Naylor *et al*., 2000; Avnimelech, 2006; Lacerda *et al*., 2006; Sena *et* al., 2006).

Extensive water exchange is a routine practice performed in shrimp culture to maintain water quality, resulting in significant dumping of nutrients and organic matter into the surrounding environment (Wang, 1990; Hopkins *et al.,* 1993; Boyd and Clay, 1998; Moss, 1999; Lacerda *et al*., 2006). These nutrients and organic matters play major role in eutrophication of aquatic environments by stimulating primary production (Thoman, *et al.,* 2001). Water is the main pathway for pathogen introduction in most of the shrimp culture systems (Lotz and Lightner, 1999). Outbreak of diseases, impacting commercial shrimp farming operations during the past two decades, has greatly affected operational management of shrimp farms worldwide (Wasielesky *et al*., 2006). Thus, crop losses due to the outbreak of diseases forced shrimp farmers to look for more bio-secure culture practices, such as minimal or zero water exchange, to minimize the risk associated with exposure to pathogens (Browdy *et al.,* 2001). Minimal water exchange technologies also enable the shrimp farming industry to avoid excessive damage to natural habitats (Naylor *et al.,* 2000; Ray *et al*., 2010).

Aquaculture systems mainly depend on the exploitation of autotrophic and heterotrophic microbial food webs. Heterotrophic food web consists of decomposition of organic matter by microorganisms, leading to the formation of assimilable detritus and inorganic nutrients. The detritus and associated microbes are directly consumed by the cultured animals or by other small animals on which the cultured species feed (Colman and Edwards, 1987; Moriarty, 1997). The heterotrophic food web consistently appears as a major contributor to the total production of the target animals (Schroeder, 1987). The fish assimilate only 15–30% of the nitrogen added in the feed in a pond environment (Acosta-Nassar *et al.*, 1994; Gross *et al*., 2000; Davenport *et al.,* 2003), the remaining quantity is lost to the system as ammonia and organic-N in the form of faeces and feed residue. The organic-N in faeces and uneaten feed undergoes decomposition resulting in ammonia production. Therefore, a high protein level in fish feed contribute to high concentration of ammonia in the water column which has detrimental to the cultured animals, and needs to be minimized.

Microbial proteins are generated in ponds with organic matter as manure or feed is decomposed by microorganisms such as bacteria and protozoa under both aerobic and anaerobic conditions. Organic matters decomposition process under aerobic condition is faster than in anaerobic condition (Reddy and Patrick, 1975). The microorganisms play major role with respect to natural productivity, nutrient cycling, water quality and the nutrition for cultured animals (Moriarty, 1997; McIntosh *et al*., 2000). In aerobic condition, microbial breakdown of organic matter leads to the production of new bacterial cell, amounting to 40–60% of the metabolized organic matter (Avnimelech, 1999). To rectify the above mentioned constraints, the biofloc technology (BFT) systems were developed to minimize effluent discharge, protect the surrounding water bodies and improve farm biosecurity (Weirich *et al.,* 2002; Burford, *et al*., 2003; Avnimelech, 2007).

The activated suspension technique (AST) recently innovated BFT is based on the use of constant and continuous aeration to allow aerobic decomposition and maintain high levels of bacterial floc suspension (Avnimelech

and Zohar, 1986; Hargreaves, 2006). Dense microbial floc developed by assimilation of inorganic nitrogen species (ammonia, nitrite and nitrate) by the heterotrophic microbial community aids in controlling water quality (Avnimelech *et al*., 1989), and the fish consume as a protein rich food source (Avnimelech *et al*., 1994). The bacterial biomass, grown on the metabolized organic substrate is taken up by the fish or shrimp as an additional food source. Nitrogen is needed to produce the protein rich microbial cells. Inorganic nitrogen is immobilized into bacterial cell when the metabolized organic substrate has a high C: N ratio. Considering the C: N ratio of bacterial cells is 5:1 (Rittmann and McCarty, 2001) and that carbon is used for respiration, the optimum C: N ratio of the organic substrates might be higher than their body composition under aerobic conditions. The C: N ratio in an aquaculture system can be increased by adding locally available cheap carbon sources and/or a reduction of protein content in the feed (Avnimelech, 1999; Hargreaves, 2006). The benefits of AST for aquaculture were described especially for shrimp culture (Burford *et al*., 2003, 2004; Wasielsky *et al.*, 2006).

BFT gives the advantages of low adverse impact on the environment due to lower external water requirements, the removal of toxic inorganic nitrogen species and the *in-situ* production of additional feed for the cultured species with high stocking density. Reduction in feed costs using BFT can be in the order of 15%. Based on investment and operation costs, nitrogen control can be performed at about half the price of normal culture practices with an external trickling filter (Schryver *et al*., 2008).

The Pacific white shrimp, *Litopenaeus vannamei* (Penaeidae) is an important shrimp which thrives well in the salinity ranges from 0‰ to 35‰ (Araneda *et al.,* 2008). It is expensive and available throughout the year. It is a hardy species having good flavour and taste. It has equal nutritional value as compared to other animal food products. It is a voracious feeder and mostly feeds on organic detritus and benthos (Cordova and Pena-Messina*,* 2005). *L. vannamei* is farmed and is well established in several countries in East, Southern, and South

Asia and plays very significant role in shrimp aquaculture production. This species is one of the candidate species for aquaculture. Farming *L. vannamei* is generally conducted extensively in grow-out ponds, and has been developed in indoor high-intensive farming system to meet the growing world demand (Lin *et al*., 2001; Zhou, 2001). With the rapid expansion and intensification, however, there is also a growing concern about the ecological sustainability of shrimp farming (Naylor *et al.,* 2000). The cultured shrimp retain only 20–30% of feed nutrient; therefore, 70–80% of high dietary protein is excreted and accumulated in water, which leads to deterioration in water quality (Avnimelech and Ritvo, 2003). Moreover, deteriorated water quality has resulted in disease outbreaks and heavy financial losses (Samocha *et a*l., 2004). Such environmental issues have created a large demand for productive, efficient and sustainable shrimp farming systems that have low impact on the environment and are more likely to be disease free (Horowitz and Horowitz, 2001). The BFT is less popular in India. Therefore, the study envisages to develop microbial biofloc for *L. vannamei* by using low-cost carbohydrate material as a carbon source to boost the production by improving the conversion of nutrients into harvestable products and maintain the water quality.

The objectives of the study were:

1. To characterize the microbial community in biofloc
2. To assess the effect of biofloc in maintaining the water quality
3. To assess the effect of biofloc on production of *L. vannamei*

2. REVIEW OF LITERATURE

Biofloc is defined as macro-aggregates composed of protozoa, diatoms, zooplankton, macro-algae, faeces, exoskeleton, remains of dead organisms, bacteria and invertebrates (Decamp *et al.,* 2003). It is the suspended growth in ponds which consists of aggregates of living and dead particulate organic matter, phytoplankton, bacteria and grazers of the bacteria (Hargreaves, 2006). It is used as natural feed for many filter feeders like *L. vannamie* and Tilapia. Biofloc is also called as microbial protein. Microbial protein has a higher availability than feed protein in the culture system (Avnimelech, 2005). Microbial density reaches 10^7 CFU ml^{-1} (Burford *et al*., 2003) forming bioflocs that contain bacteria and other microorganisms.

2.1. Biofloc technology

Biofloc technology is an aquaculture systems were heterotrophic bacteria and algae are grown in flocs under controlled conditions within the culture system (Avnimelech, 2006). BFT is the utilization of microbial processes within pond itself to treat water and provide food resources for cultured animals. The system is based on the knowledge of conventional domestic wastewater treatment systems and is applied in aquaculture. Biofloc technology system also called as active suspension ponds, heterotrophic ponds and green soup ponds etc.

2.2. Concept of biofloc technology

Biofloc technology is based on the carbon nitrogen ratio maintained by adding carbohydrates into the system. Knoesche and Tscheu (1974) adopted the idea to utilize the heterotrophic bacteria as a natural supplement feed for growth of shrimp and fishes in aquaculture systems. They used activated sludge to process and treat water in a recirculation system, and proposed to mix produced sludge with grains for later re-use as fish feed for carps. The carbohydrate is added for immobilization of ammonia excreted by the cultured

animals (Avnimelech and Lacher, 1979; Boyd, 1985; Muthuwani and Lin, 1995). If carbon and nitrogen are well balanced in the solution, ammonium in addition to organic nitrogenous waste will be converted into bacterial biomass (Schneider *et al*., 2006). Bacterial growth is stimulated by adding carbohydrates and nitrogen uptake through the faeces of cultured animal for the production of microbial proteins (Avnimelech, 1999). The promotion of nitrogen uptake by bacterial growth decreases the ammonium concentration more rapidly than nitrification (Hargreaves, 2006). Immobilization of ammonium by heterotrophic bacteria occurs much more rapidly because the growth rate and microbial biomass yield per unit substrate of heterotrophs factors are 10 times higher than that of nitrifying bacteria (Hargreaves, 2006). The principles of growing fish or shrimp in limited water exchange intensive ponds were developed simultaneously for shrimp in the Waddel Mariculture Centre, USA and for fish (Tilapia) in Israel (Avnimelech *et al*., 1989, 1994; Hopkins *et al*., 1993; Chemberlain and Hopkins, 1994) and practiced in USA (Serfling, 2000), in the beginning of the 1990's. The microbial biomass yield per unit substrate of heterotrophic bacteria is about 0.5 g biomass C/g substrate C used (Eding *et al.,* 2006).

2.3. Importance of biofloc technology

Biofloc technology system is used to reduce effluent discharge, protect the surrounding water bodies, avoid outbreak of diseases and improve farm biosecurity (Burford *et al.*, 2003; Avnimelech, 2007). The microorganisms play major roles with respect to natural productivity, nutrient cycling, water quality and the nutrition of the cultured animals (Moriart, 1997; McIntosh, 2000). Control of the heterotrophic bacterial community over autotrophic microorganisms is achieved predominantly by the use of high carbon to nitrogen ratios (C: N) (Avnimelech *et al*., 1994). The uptake of ammonia by bacteria improves the water quality and increases microbial biomass production (Avnimelech, 1999; Moss, 1999). These are the important factors which serve as fuel for practicing the "floc system" (Burford *et al*., 2004; Cohen *et al.,* 2005). Due to availability of a nutrient-rich feed source throughout the day, there is reduction in the artificial feed inputs

and costs (Browdy *et al*., 2001; Avnimelech, 2007; Samocha *et al*., 2004). With respect to feeding, this technique operates at "neutral cost", because it upgrades starch to protein. Moreover, one does not need to invest in an external water treatment system. This method is applicable to extensive as well as intensive aquaculture systems. In addition, the heterotrophic microbial biomass is believed to have a controlling effect on pathogenic bacteria (Michaud *et al.,* 2006). Presence of poly-β-hydroxybutyrate (PHB) in bioflocs accumulating bacteria may abate pathogenic bacteria in aquaculture (Defoirdt *et al.,* 2007; Halet *et al.,* 2007). Biofloc technology has shown many advantages for nursery-reared, post-larval Malaysian prawns, including maintenance of optimal water quality, higher survival rate, less expenditure for water and control of waste, higher nutritional quality, and smaller impacts on the environment (Perez-Fuentes *et al*., 2013).

2.4. Selection of culture species

The species selection is one of the major criteria that decide the success of the BFT in aquaculture (Avnimelech*,* 2007; Azim and Little, 2008). Not all species are candidates to BFT. Some characteristics seem to be necessary to achieve a better growth performance such as resistance to high density, tolerance to intermediate levels of dissolved oxygen (~3-6 mg L^{-1}), settling solids (~10 with a maximum of 15 ml L^{-1} of "biofloc volume", measured in Imhoff cones) in water (Taw*,* 2010) and N-compounds, presence of filtering apparatus (i.e. tilapia, *L. vannamei*), omnivorous habits and/or digestive system adaptable to better assimilate the microbial particles. The cultured animal should have the ability to harvest the bacterial flocculates developed in the system, and should have the ability to digest and utilise the microbial protein. *L. vannamei* are capable of ingesting and retaining nitrogen derived from natural biota such as bacteria and algae (Burford *et al*., 2004). An excessive turbidity may cause negative effects on sensitive shrimp species and not all the species are adaptable to growing in turbid water (Avnimelech*,* 2006).

BFT is proved to be beneficial for both shrimp and finfish culture (Milstein *et al.,* 2001; Burford *et al.,* 2003; 2004; Serfling *et al*., 2006; Wasielesky *et al*., 2006, Widanarni *et al*., 2012). Tilapia was the first species studied with biofloc in ponds, the nibbling habit and feeding behaviour were the reasons for the selection of this species. Later, marine shrimps (*L. vannamei, Penaeus semisulcatus, Marsupenaeus japonicus, Farfantepenaeus paulensis)* were also identified as the candidate species for BFT aquaculture (Burford *et al*., 2003, 2004; Wasielesky *et al.,* 2006; Hari and Lorenzen*,* 2004; Hari *et al*., 2006; Arnold *et al.,* 2009; Kuhn *et al.,* 2010; Ballester *et al.,* 2010; Megahed, 2010; Emerenciano *et al*., 2011; Zhao *et al.*, 2012).

2.5. Biofloc production methods

Nitrogen removal from aquaculture pond water by heterotrophic nitrogen assimilation in lab-scale sequencing batch reactor was studied by Schryver and Verstraete (2009). Study by Kuhn *et al*. (2009) showed that bioflocs harvested from the sequencing batch reactors (SBRs) and membrane biological reactors proved to be a suitable and often superior ingredient, to soybean protein and fishmeal in lab-scale feeding trials with *L. vannamei.*

2.6. Biofloc components

Microbial floc generally consists of filamentous bacterial particles, protozoans, zooplankton, colloids, organic polymers and dead cells surrounded by a gelatinous matrix, which contains extra-cellular polymeric substances that encapsulate the microbial cells and play a major role for flocculation of floc. (Jorand *et al.,* 1995; Avnimelech, 2007). Ray *et al.* (2010) reported that a biofloc particle contained some of the microbial components, high abundance chlorophytes, three types of diatoms, heterotrophic dinoflagellates, a nematode and a rotifer etc. The grouping of bacteria within the floc mainly depends upon the zeta potential and Van der Waals forces (Sobeck and Higgins, 2002).

2.7. Biofloc structures

Microbial flocs consist of a heterogeneous mixture of microorganisms (floc-formers and filamentous bacteria), particles, colloids, organic polymers, cations and dead cells (Jorand *et al.,* 1995) and can reach more than 1000 µm in size. Typical flocs are irregular by shape, having a broad distribution of particle sizes are fined, easily compressible, highly porous (up to more than 99% porosity) and permeable to fluids (Chu and Lee *et al*., 2004). Individual bacterial cells are sized in the order of 1 µm (Madigan and Martinko, 2006). This implies that these organisms are in general surrounded by a layer of liquid that hampers the mass transfer of nutrients and waste products (Logan and Hunt, 1988). The floc contains only 2–20% live microbial cells, total organic matter 60–70% and total inorganic matter 30–40% (Wilen *et al*., 2003). The main constituents that can be found within the floc matrix are the extracellular polymeric substances. These structures form a matrix that encapsulates the microbial cells, and play a major role in binding the floc components together. The presence of these structures in activated sludge systems can be substantial, up to 80% of the total mass (Hantula and Bamford, 1991; Liu and Fang, 2003). They are typically made up of polysaccharides, protein, humic compounds, nucleic acids and lipids (Zita and Hermansson, 1994). They are produced as slime or capsule layers under various nutritional conditions but particularly in case of limitation by nutrients like e.g. nitrogen (Steiner *et al.,* 1976).

2.8. Nutritional quality of biofloc

Biofloc quality was independent of the quality of feed applied and contained more than 50% crude protein, 2.5% crude lipid, 4% fibre, 7% ash and 22 kJ g^{-1} energy on dry weight basis (Azim *et al.,* 2008). The crude protein content of biofloc collected from BFT treatments was within the range of 35-53% (Azim *et al*., 2008; Schryver and Verstraete, 2009; Crab *et al*., 2010; Kuhn *et al*., 2010; Ekasari *et al*., 2010). Kuhn *et al.* (2010) evaluated the proximate and mineral content of two types of biofloc produced in membrane biological reactor

(MBR) and sequencing batch reactors (SBR). In grow-out system protein requirement of *L. vannamei* were reported to be between 30% and 44%, depending on the study, and the size of the shrimp (Guillaume, 1997; Rosas *et al.,* 2001). Biofloc has sufficient amount of protein (35-50%) and trace minerals for the growth of the shrimps. Flocculated particles in these systems contribute substantially to shrimp nutrition (Hopkins *et al*., 1995; McIntosh and Avnimelech, 2001). Essential amino acid as well as other essential feed components was found in the flocs at ample levels (Tacon *et al.,* 2002; Decamp *et al*., 2003). Microbial flocs contained vitamins and trace metals at levels enabling to omit the addition of these growth factors to the feed and thus saving about 30% of the feed cost.

Nutritional composition of biofloc differs according to environmental condition, carbon source applied, TSS level, salinity, stocking density, light intensity, phytoplankton and bacteria communities and ratio, etc. Regarding to age of bioflocs, in "young" biofloc, heterotrophic bacteria is mainly present as compared to "old" biofloc dominated by fungi (Kuhn and Lawrence, 2012). In biofloc particles, protein, lipid and ash content could vary substantially (12 to 49, 0.5 to 12.5 and 13 to 46%, respectively. The same trend 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) influence FA, Vitamin and amino acids profile (Logan *et al*., 2010).

2.9. Carbon nitrogen ratio

The C/N ratio is a potential element for controlling inorganic nitrogen in the aquaculture systems. A concentration of about 10 mg NH_4^+-N L^{-1} could

almost completely be removed within 5 h after the addition of glucose at C/N ratio 10, and without the accumulation of nitrite and nitrate (Avnimelech, 1999). Nitrogen is needed to produce protein rich microbial cells. Inorganic nitrogen is immobilized into bacterial cell when the metabolized organic substrate has a high C: N ratio. Considering the C: N ratio of bacterial cells to be 5:1 (Rittmann and McCarty, 2001), carbon is used for respiration, the optimum C: N ratio of the organic substrates might be higher than their body composition under aerobic conditions. The C: N ratio in an aquaculture system can be increased more than 10:1 by adding different locally available cheap carbon sources and/or a reduction of protein content in feed (Avnimelech, 1999; Hargreaves, 2006). Accumulation of toxic inorganic nitrogen species (NH_4^+, NO_2^-) is prevented in bioflocs system by maintaining a high C/N ratio and inducing the uptake of ammonium by the microbial community (Avnimelech *et al*., 1994; McIntosh *et al*., 2000).

2.10. Factors influencing biofloc formation

There are several factors that influence floc formation and floc structure (Schryver *et a*l., 2008). Ritvo *et al*. (2003) studied the effect of salinity and pH on the colloidal properties of suspended particles in super intensive aquaculture systems. The mixing intensity imposed by a selected aeration device at a certain power input will determine the steady-state of floc size. It is equilibrium between the rate of aggregation and the rate of breakage and floc size distribution (Spicer and Pratsinis, 1996; Chaignon *et al.,* 2002). Dissolved oxygen also influences the biofloc formation. Previous studies showed that biofloc produced at lower dissolved oxygen levels have a higher floc volume index (FVI). The organic carbon sources of choice, to a large extent determine the composition of the floc produced (Mikkelsen *et al*., 1996; Hollender *et al.,* 2002; Oehmen *et al.,* 2004). Organic loading rate, temperature and pH are the other factors which also governs the biofloc formation (Schryver *et al.,* 2008). pH has a significant role in the floc formation both qualitatively and quantitatively in the culture of *P. monodon*, pH of 7.5 is found to be optimum for better biofloculation in shrimp aquaculture (Devi, 2009; Kurup and Devi, 2010).

2.11. Carbon source and their requirements

The carbon sources applied in BFT are often by-products derived from human and/or animal food industry, preferentially localy available. Cheap sources of carbohydrates such as molasses, glycerol and plant meals (i.e. wheat, corn, rice, tapioca, etc) will be applied before fry/post-larvae stocking and during grow-out phase, aiming to maintain a high C:N ratio (~15-20:1) and to control N compounds peaks. Also, a mix of plant meals can be pelletized ("green-pellet") and applied into ponds (Taw, 2010); or low protein diets containing high C: N ratio can also be carried out (Azim *et al.*, 2008; Avnimelech, 2012). The carbon source serves as a substrate for operating BFT systems and production of microbial protein cells (Avnimelech, 1999). There are many considerations for its selection such as costs, local availability, biodegradability and efficiency of bacteria assimilation.

Schneider *et al*. (2006) used molasses as carbon source for heterotrophic bacteria production on solid fish waste. Crab *et al*. (2010) used different carbon sources for studying the nutritional value of biofloc, were grown on acetate, glycerol and glucose in the culture system of *M. rosenbergii* post-larvae. Varghese (2007) evaluated performance of various locally available various carbohydrate sources for biofloculation of the culture system of *P. Monodon*; tapioca flour was the biofloculating agent chosen by Hari *et al.* (2004, 2006), Varghese (2007), and Kurup and Varghese (2007a, 2007b). The microbial protein were developed using two diets having the crude protein level of 35% (C: N = 8.4) and 22% (C: N = 11.6), fed with wheat flour (Azim *et al.,* 2008). The bioflocs were produced commercially available sequencing batch reactor using sucrose as a carbon source (Kuhn *et al*., 2009). In this system most driving force was heterotrophic bacteria which consumed organic carbon for their intensive growth. For 1.0 g of carbohydrate-C yields about 0.4 g of bacterial cell dry weight-C; and depending on the bacterial C/N-ratio they immobilize mineral nitrogen. As such, Avnimelech (1999) calculated 20 g of carbohydrate was needed to immobilize 1.0 g of N, based on a microbial C/N-ratio of 4 and a 50% C in dry carbohydrate.

2.12. Effect of biofloc on the growth of cultured species

The microorganisms in biofloc partially replace protein content in shrimp diets but this is not always the case (McIntosh and Avnimelech, 2001; Wasielesky *et al*., 2006). Recent studies showed that reducing the protein content of diet would affect growth performance of shrimp reared in biofloc conditions. Ballester *et al*. (2010) found that at least 10% of protein content in pelletized feed could be reduced when *Farfantepenaeus paulensis* postlarvae were raised in BFT conditions. Nunes *et al*. (2010) 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 more 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* in a relatively low temperature (25°C) under biofloc conditions (Emerenciano *et al.,* 2011). The results showed that it was possible to replace 1/3 part of a conventional diet by alternative low-protein biofloc meal pellet without interfering survival and shrimp performance.

Azim and Little (2008) reported 100%survival and net yield 45% higher in the biofloc tanks than in the control tanks, obtained in *Oreochromis niloticus* farming when biofloc was taken by fish as food. The microorganisms not only remove excess nutrients, but have been implicated in nutritional provision for animals, including shrimp and tilapia, that can result in improved growth rate, feed conversion ratio (FCR) and weight gain (Moss and Pruder, 1995; Burford *et al.,* 2004; Wasielesky *et al.,* 2006; Azim and Little, 2008). This process was quantitatively formulated (Avnimelech*,* 1999), verified and practiced by farmers world-wide (Browdy *et al.,* 2001; Panjaitan, 2004). Addison *et al.* (2010) studied the effect of biofloc in replacement of fish meal and soybean meal in semi-purified shrimp diets. Ju *et al*. (2008) studied the enhanced growth effect on shrimp (*L. vannamei*) through feeding of floc or floc fractions incorporated in formulated shrimp diet. The result showed that inclusion of the floc material in shrimp diets

could enhance the shrimp growth. Shrimp preferred the experimental diets than the commercial diet and the feed stability was same as that of commercial diet. Addison *et al.* (2010) reported increased growth of *L. vannamei* through feeding of biofloc by replacing fishmeal and soybean. Microbial flocs produced in suspended growth bioreactors could offer the shrimp industry a novel alternative feed. Biofloc provided as a feed for different cultured species such as Nile tilapia (*Oreochromis niloticus*) and white-leg shrimp, *L. vannamei* (Azim and Little, 2008; Crab *et a*l., 2009; Kuhn *et al*., 2009). Burford *et al*. (2004) found that "flocculated particles" rich in bacteria and phytoplankton could contribute substantially to the nutrition of *L. vannamei* in intensive shrimp ponds.

Studies have shown that growth rates of *L. vannamei* grown in pond water were higher than in water with a low load of particulate organic matter (Leber and Pruder, 1988; Moss and Pruder, 1995; Otoshi *et al*., 2001). The presence of biofloc structures with epiphytic growth increased *L. vannamei* production (Bratvold and Browdy, 2001). In semi-intensive *L. vannamei* and *Penaeus setiferus* ponds, 52% of the retained nitrogen was supplied by the pond biota (Parker and Anderson, 1989; Parker *et al*., 1991). The consumption of detritus matter has previously been shown for other shrimp species (Moriarty and Barclay, 1981; Focken *et al*., 1998). Two types of microbial flocs, derived from biological treatment of fish effluent, were compared by incorporating in the feed of *L. vannamei* (Kuhn, 2010).

Experimental trials showed that microbial flocs of different sizes could be taken up by fish or shrimp and served as a protein rich feed (Tacon *et al*., 2002; Burford *et al*., 2004) which would help to reduce the cost of production by reducing the protein content of the artificial feed and improving the overall economics of the culture system (McIntosh, 1999; Moss, 2002). Bio-flocs do not allow complete replacement of the traditional food but still can bring about a substantial decrease of the processing cost since the food represents 40–50% of the total production costs (Craig and Helfrich, 2002).

2.13. Microbial community in biofloc

The most abundant microbes in the floc particles are chlorophytes (green algae), diatoms, dinoflagellates, nematodes, rotifers, and cyanobacteria (DeVantier *et al*., 1998; Ray *et al*., 2010). Heterotrophic ammonia-assimilative and chemoautotrophic nitrifying bacteria are primarily responsible for maintaining water quality and lead to minimal-exchange in intensive systems (Ebeling *et al.,* 2006; Hargreaves, 2006). The microorganisms play important roles in nutrient cycling such as methane production (Chan *et al*., 2005), sulphate reduction (Li *et al*., 1999) and ammonia oxidation (Hastings *et al.*, 1998) in natural aquatic system. Microbiological aspects particularly bacterial growth patterns, characterization of biofloc and possible manipulation of microbial community is necessary for successful design and operation of biofloc technology (Azim *et al*., 2008).

The most easy and common method to determine the presence and type of microorganisms in a sample is microscopy. Since the method is based on visual morphology it is generally not possible to identify all of them. It can be used to gain a value of the proportion of filamentous and zoogloeal flocs within a water sample. The fluorescence in-situ hybridization (FISH) procedure is based on the binding of fluorescently labelled DNA probes with the ribosomal RNA of bacteria (Amann *et al.,* 1995). The DNA probes can be designed to exclusively bind to rRNA of a chosen type of microorganism and thus allow detecting a certain species in a community. Since rRNA is present only in biologically active organisms, it only allows to detect the ones that are performing a specific task (non-active cells are not detected). Real-time polymerase chain reaction (PCR) is a molecular technique that allows simultaneous amplifying and quantifying of the extracted DNA from a sample (Heid *et al*., 1996). This technique is commonly used to determine the amount and type of microorganisms present in a sample. A quantitative array allows simultaneous quantification of phylogenetic and functional genes involved in the activity of interest, e.g. nitrification and denitrification processes (Geets *et al*., 2007). As such, the evolvement of a complete system can be analysed.

Denaturing gradient gel electrophoresis (DGGE) is a molecular approache that provide information about the genetic microbial diversity in sample such as water, sludge, air, etc. (Muyzer *et al*., 1993). This technique is based on the separation of extracted DNA and by PCR amplified genes (mostly 16S rRNA genes), unique for a group of microorganisms. The analysis of an environmental sample by means of DGGE results in a band pattern in which roughly each band represents a specific microorganism. The information that can be obtained from a DGGE band pattern is limited, except for comparative purposes. For instance, only bacteria that are present at more than 1% of the total community are detected. The technique is mostly used as a research tool to visualize shifts in the microbial population composition in time. Relatively new concepts that offer the possibility to make use of the DGGE band patterns in an alternative way comparing the bacterial diversity in biofloc. Moving window analysis is a technique based on DGGE to detect shifts in the microbial community in time (Wittebolle *et al.,* 2005). By means of Bionumerics software (Applied Maths, Sint-Martens-Latem, Belgium), DGGE patterns can be analysed and compared, thereby quantifying the differences. Both techniques can be applied in BFT. Relations between the shifts in microbial populations and changes in performance may be established.

Studies employing denaturing gradient gel electrophoresis (DGGE), to compare the diversity of 16S rRNA gene amplicons obtained directly from samples, are very less. DGGE method can clearly indicate the existence of common groups of microorganisms in natural aquatic system, but it does not permit more precise identification of these microbes to the species or sub-species level. DGGE has proved to be an exceptional tool to study species diversity and bacterial community dynamics. But a more comprehensive understanding of the physiology of these organisms and their complex biogeochemical processes will require their cultivation, isolation and characterization (Bae *et al*., 2005). It is well known that more than 90% of the microorganisms existing in nature are refractory to selective enrichment cultures (Ward *et al.,* 1990). To overcome the constraints of these culture-dependent methods, researchers currently focus on the use of molecular biological techniques, with their powerful capacity to allow the analysis

of microorganisms in their natural habitats. In this context, analysis of the 16S rRNA gene is the most widely used approach in the last decade. Of the 16S rRNA gene-based methods used for studying complex microbial populations, DGGE has received the most attention and has been successfully applied to several natural habitats. Profiling of microbial communities from soil, marine environments, hydrothermal vents, the gastro-intestinal tract of humans and even the study of a microbial community resident in a medieval wall painting (Felske *et al.,* 1997; Hold *et al.,* 2001; Nicol *et al.,* 2003) has already been done using DGGE technique. More recently, DGGE has been introduced into food microbiology for the identification of microorganisms isolated in food and for evaluation of the microbial diversity during food fermentation. Ray *et al.* (2010) characterized microbial communities in minimal exchange, intensive aquaculture systems and studied the effects of suspended solids management. Characterization of microbial community structure in shrimp biofloc cultures using biomarkers and analysis of floc amino acid profile (Ju *et al*., 2008; Ray *et al*., 2010). The predominant microbe analysed with denaturing gradient gel electrophoresis (DGGE) was characterized by *Bacillus* sp. in the bioflocs treatment group, but by *Vibrio* sp. in the relative control group (Zhao *et a*l., 2012).

2.14. Water quality management

Water quality is the key factor for the success of aquaculture and that ensures the survival, production and growth rate of the cultured animals (Boyd*,* 1990; Burford, 1997). Biofloc technology considered a culture technique in which water quality is maintained and in situ animal feed is simultaneously produced in the form of biofloc particles (Crab *et al.,* 2007). Water quality management in the biofloc system is based upon developing and controlling dense heterotrophic bacteria within the culture component (Avnimelech*,* 2007). The control of inorganic nitrogen accumulation in the culture system is based upon carbon metabolism and nitrogen immobilization into microbial cells and they are composed of proteins (Avnimelech *et al*, 1989; Avnimelech, 1999; Crab *et al*.,

2007). The addition of fertilizers or carbon sources directly to the pond increase the natural productivity (Crab *et al.,* 2007; Uddin *et al.,* 2007).

Biofloc technology also has potential to protect the cultured organisms from infections with pathogenic bacteria, which are responsible for spreading of diseases which causing major economic losses in aquaculture (Crab *et al*., 2010). Biofloc can be a novel strategy for disease management on a long-term basis, in contrast to conventional approaches such as antibiotic, probiotic and prebiotic application (Sinha *et al*., 2008). Recent research focused on the value addidion of bioflocs, specifically regarding pathogen control, because infectious diseases destroy the aquaculture industry. It was observed that the regular addition of carbon to the culture media is known to select for poly-hydroxyl alkanoate (PHA) accumulating bacteria (Salehizadeh and Von Loosdrecht, 2004) such as *Alcaligenes eutrophus, Azotobacter vinelandii*, *Pseudomonas oleovorans* and others that synthesise PHA granules. Such granules are synthesised when an essential nutrient like nitrogen is limited in the presence of an excess carbon source (Avnimelech, 1999). 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 breakdown of PHA in the gastrointestinal tract can be carried out via enzymatic and chemical hydrolysis (Yu *et al*., 2005). These compounds are capable of exhibiting bacteriostatic and or bacteriocidal properties, depending on the physiological status of the host and the physico-chemical characteristics of the external environment (Ricke, 2003). Crab *et al.* (2010) studied the use of bioflocs as new bio-control agents for aquaculture using a model system with brine shrimp (*Artemia franciscana*). They found that the bioflocs can also be used as a feed for brine shrimp (*A. franciscana*) nauplii. The technique was also applied in the nursery rearing of *P. monodon* with an addition of carbon source and artificial substrates. They found that biofloc influences positive growth and production of shrimp juveniles, in addition to providing more favourable water quality conditions irrespective of higher stocking densities (Arnold *et al*., 2009).

2.15. Environmental problems through aquaculture

Aquaculture production has increased on an average annual rate of 8.9% since 1970, as compared to an annual growth rate of 1.2% and 2.8% for capture fisheries and terrestrial farmed meat production over the same frame. To overcome the demands, aquaculture production must grow by five fold in the next five decades. This development has to overcome three major constraints: a) Produce more fish without significantly increasing the use of basic natural resource of water and land, b) Develop sustainable systems that will not damage the environment, c) Develop systems providing a reasonable cost/benefit ratio, to support the economic and social sustainability of aquaculture (Avnimelech, 2012).

Need was felt to adopt eco-friendly technologies to reduce the adverse impacts of aquaculture on social and physical environments (Alagarswami*,* 1995). Intensive aquaculture systems efficiently produce dense biomasses of fish or shrimp and they were faces two major problems. The first is the water quality deterioration caused by the high concentrations of metabolites and the second is the low feed utilization in cases when high water exchange, within or outside the pond system (Avnimelech, 2007). Artificial formulated feed is the major investment in aquaculture and it constitutes 40-60% of operational cost in shrimp production (Mitra *et al.,* 2005). The major portion (>80%) of artificial feed is lost in the aquaculture system as uneaten feed and faeces (Daniels and Boyd, 1989; Siddiqui and Al-Harbi, 1999; Rahman, 2006). The uneaten feed in the system lead to deteriorating the water quality through decomposition (Horner *et al*., 1987; Poxton and Allouse, 1987; Cowey and Walton, 1989; Poxton and Lloyd, 1989; Moreira *et al*., 2008). Because of the higher dietary protein in the feed it deteriorates the water and soil qualities in shrimp grow-out ponds (Boyd, 1989). Success or failure of the aquaculture operation was wholly depending on the water quality and water source. While selection of a suitable water source the physical and chemical characteristics such as suspended solids, temperature, dissolved gases, pH, mineral content and the potential danger of toxic metals

must be considered for the success of aquaculture operation (Boyd and Tucker, 2009).

The shrimp aquaculture have proven to be destructive to the natural environment and population of aquatic animals (Gowen and Bradbury, 1987; Folke *et al*., 1994; Kautsky *et al.,* 1997; Naylor *et al.,* 2000; Milewski, 2001). Crustacean aquaculture is fraught with environmental problems that arise from: (i) the consumption of resources such as land, water, seed and feed; (ii) their transformation into products valued by society; and (iii) the subsequent release wastes into the environment (Kautsky *et al*., 2000; Ronnback, 2001). The direct impacts include release of nutrient rich water and toxic chemicals, the transfer of diseases and parasites to wild stock. It can also impact the loss of habitat and niche space, and changes in food webs. In semi intensive and intensive farms, artificial feeds provide most of the nitrogen (N), phosphorus (P) and organic matter inputs to the pond system. Only 17% (by dry weight) of the total amount of feeds applied to the pond is converted into shrimp biomass (Primavera, 1993). The rest is leached or otherwise not consumed, egested as faeces, eliminated as metabolites, etc. leads to the eutrophication.

The benefits of BFT has been described extensively for shrimp culture (Buford *et al.,* 2003, 2004; Hari *et al.,* 2004_a, 2006; Wasielesky *et al*., 2006) and for finfish culture (Avnimelech, 1999; 2007; Milstein *et al*., 2001; Serfling, 2006). Application of BFT making culture more sustainable by reduce water based nitrogen species discharge to the environment ,reducing the protein content of the feed and increase of total revenue (54%) from the harvested shrimp, (Kurup, 2009). Enhanced pond productivity through stimulation of suspended and attached algal development and by using them to improved water quality, provide additional food, higher carrying capacity in combination with reduced nutrient discharge, improving environmental sustainability. This technology is simple and cheap, making it also socially and economically sustainable, even for small scale (Hari *et al*., 2004_b, 2006; Varghese, 2007; Kurup, 2009).

2.16. Status of biofloc aquaculture

Biofloc technology has widely applied in the farming of Tilapia, *P. monodon*, *L. vannamei* and *M. rosenbergii*. It was commercially first applied in Belize by *Belize Aquaculture* (North America). It also has been applied with success in shrimp farming in Indonesia and Australia (Taw, 2010). The combination of two technologies, partial harvesting and biofloc, has been studied in northern Sumatra, Indonesia. The numbers of shrimp farms currently using biofloc technology are *Belize Aquaculture Ltd*., in Belize and *P.T. Central Pertiwi Bahari* in Indonesia. *Belize Aquaculture* was the first commercial farm to use biofloc technology successfully. Its production of 13.5 MT shrimp ha^{-1} was quite an achievement at that time. The Belize technology was applied initially in Indonesia at C.P. Indonesia (now P.T. Central Pertiwi Bahari, C.P. Indonesia), which achieved average production over 20 MT ha^{-1} in commercial 0.5-ha lined ponds. Research trials reached 50 MT ha^{-1}. The technology combined with partial harvest was repeated in Medan, Indonesia, with better results. During 2008 and 2009, biofloc technology was used in Java and Bali successfully.

In Indonesia, biosecurity protocols were incorporated within the technology. The correct number and position of paddle-wheel aerators used in ponds are essential for the success of this technology (Taw, 2010). In China a number of shrimp farmers are adopting this technology and their fully HDPE-lined, plastic-covered shrimp grow-out ponds with high density culture are ideal for the technology. A group from Brazil is running commercial biofloc trials. Malaysia is currently initiating a 1,000-ha integrated intensive shrimp-farming project at Setiu, Terengganu by Blue Archipelago. This technology was first applied in the extensive culture system of *P. monodon*, in Kerala, by School of Industrial Fisheries, Cochin University of Science and Technology (Hari *et al*., 2004_a, 2006). BFT is also applied with success in the hatchery system of freshwater prawn, *M. rosenbergii* (Saritha, 2009). This technology also applied in the hatchery phase of *P.monodon* (Devi and Kurup, 2011).

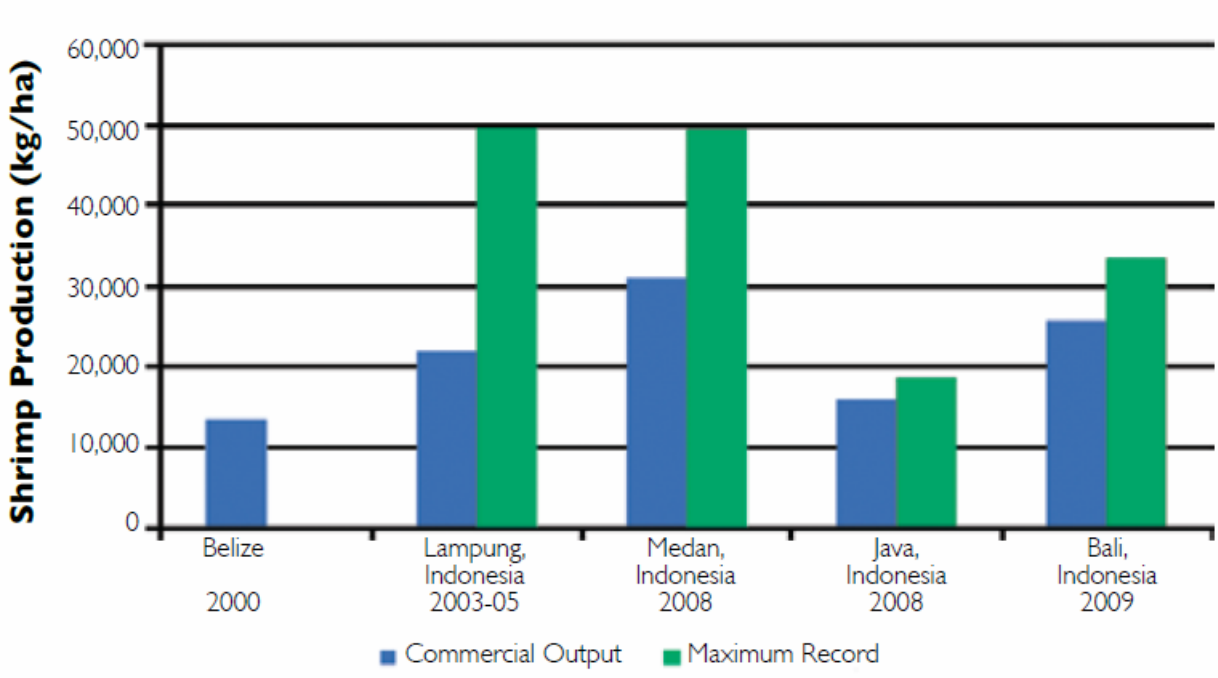

Fig. 1. Shrimp production levels at various farms implementing biofloc technology (Taw *et al.*, 2010)

3. MATERIALS AND METHODS

The study was conducted to compare the effects of microbial biofloc on water quality, changes in microbial diversity and growth of the Pacific white shrimp (*Litopenaeus vannamei*) fed with different carbon sources. The sampling was carried out at fortnightly interval to investigate the growth, microbial and water quality parameters of biofloc system.

3.1. Site of the Experiment

The experiment was conducted over a period of 120 days from 1st October 2012 to 30thJanuary 2013 at Wet Laboratory of the Central Institute of Fisheries Education (CIFE), Versova, Mumbai.

3.2. Experimental design

FRP circular tanks having capacity of 500 L and diameter of 0.98 m were used for this experiment. Three treatments with a control in triplicates were set up by completely randomized design (CRD). Three treatments were biofloc tank fed with sugarcane molasses (BFT_S), BFT fed with tapioca flour (BFT_T) and BFT fed with wheat flour (BFT_W). All the treatments and control were fed with commercially available feed (Charoen Pokph and (India) Pvt. Ltd., India) having 37.5% CP twice in a day.

3.3. Biofloc production

Uniform sizes of circular FRP (fiber reinforced plastic) tanks of 500 L capacity were used for biofloc production. The tanks were initially washed with and filled with potassium permanganate solution (4 mg L^{-1}) for overnight and thereafter were thoroughly washed with clean water and were allowed to dry under the sun. The vigorous aeration was provided in all the experimental tanks from a centralized aeration unit. The aeration pipe in each tank was provided with an air stone and a plastic regulator to control the air pressure in all tanks.

The seawater was collected from the Aksa Beach (Mumbai, India) during high tide time. The collected seawater was stored in the 5000 L reservoir tank. The seawater was allowed to settle down for a week and was diluted with freshwater to make a salinity of 25 ‰. The diluted seawater (25 ‰) was filled in the experimental tanks. The biofloc was produced for 350 L water in a 500 L capacity tanks before stocking of seeds. The sugarcane molasses were prepared by fermenting a boiled sugarcane juice with commercially available yeast for 2 days. After two days it was ready to be used as input for biofloc production. Tapioca flour and wheat flours were purchased from the local market. Sugarcane molasses, tapioca flour and wheat flour were added as a carbon source to respective treatment tanks as per the calculation shown in section 3.4. Feed was applied at a rate of 20 g in each tank every day. By adding carbon source, the C: N ratio approximately 20:1 was maintained throughout the experiment to optimize heterotrophic bacterial growth (Avnimelech, 1999; Asaduzzaman *et al*. 2008).

3.4. Quantity of carbohydrate required for biofloculation

The quantity of carbohydrate added to the BFT system was calculated using the equation suggested Avnimelech (1999) and assuming that the added carbohydrate contains minimum of 50% carbon, the CH addition needed (ΔCH) to reduce the total ammonia nitrogen concentration by 1g N m^{-3} is 20 g $m^{-3.}$

$$\Delta CH = \Delta N / 0.05 \quad \text{---------- (1)}$$

It can be assumed that the ammonium flux into water, $\Delta NH_4^{+,}$ directly by excretion or indirectly by microbial degradation of the feed residues, is roughly around 50% of the feed nitrogen (Avnimelech, 1999).

$$\Delta N = \text{quantity of feed} \times \%\ \text{N in feed} \times \%\text{N excretion} \quad \text{---------- (2)}$$

The amount of carbohydrate addition needed to assimilate the ammonium flux into microbial protein was calculated using equations (1) and (2):

$$\Delta CH = \text{quantity of feed} \times \%\ N \text{ in feed} \times \%\ N \text{ excretion}/0.05 \text{------ (3)}$$

Shrimp were fed with commercial feed pellets containing 37.45% protein at a daily rate of 1.5% body weight. It was assumed that 33% of the feed nitrogen is excreted. According to equation (3), 599 g of carbohydrates is required for each kg of 37.45% protein feed.

3.5. Stocking of shrimp seed and tank management

The specific pathogen free (SPF) *L. vannamei* (PL9) seeds were procured from a Madha hatchery, Chennai. The seeds were given dip treatment with $KMnO_4$ solution for about 5-10 seconds to avoid any possible contamination or to avoid transmission of disease. The seeds were nursed in 500 L tanks for two weeks before being stocked in the experimental tanks. The seeds (20days old larvae) were stocked when bioflocs (measured as total suspended solids TSS and floc volume) was higher than 100 mg L^{-1} and between 5-50 ml. The shrimps were stocked into 30 days old biofloc treatments and control tanks at the rate of 130 PL m^{-2}. Tanks were aerated with an air pump to maintain dissolved oxygen content in the saturation level.

CP manufactured Pelleted feeds (1.8-3 mm) were used throughout the experiment. Proximate composition of the experimental diet and the carbon sources are given in Table 1. Feeding rates were based on observation of feeding behaviour of shrimp in the biofloc treatments during the first few days and fixed at 1.5% of the total stocked biomass daily, and adjusted fortnightly after weighing shrimp sample. The same amount of feed was fed to all the treatment and control tanks. Daily feed rations were split into two equal amounts given at 0800 and 1700 h to all the tanks.

Table 1. Proximate composition of experimental feeds and carbon sources based on dry weight basis (g/100g)

Constituent (%)	Feed	Sugarcane molasses	Tapioca flour	Wheat flour
Crude protein	37.45	6.30	11.46	14.72
Ether Extract	12.32	4.01	3.19	3.25
Crude fiber	3.27	3.75	6.16	5.15
Total ash	9.80	16.20	5.10	2.42
Nitrogen free extract	37.16	69.74	74.04	74.46
Moisture	5.35	22.67	8.26	6.64

3.6. Media, chemicals, Glassware, Plasticware and other Research Material

Chemicals and reagents were procured from Merck, Mumbai; S. D. Fine Chemicals, Mumbai; Fisher Scientific, Mumbai; Qualigens Fine Chemical, Mumbai; High Purity Laboratory Chemicals Pvt. Ltd., Mumbai and Sigma, USA. The bacteriological agar media and agarose were procured from Hi-media. The PCR master mix, DNA molecular weight markers, 6x loading dye and other molecular biology grade chemicals were procured from Genei Pvt Ltd, Bangalore and MBI Fermentas Life Sciences, Lithuania. The primers were designed from Eurofins Genomics Pvt Ltd, Bangalore. The glassware and plasticware used in this study were procured from Borosil, Tarson and Merck, Mumbai.

3.6.1. Sterilization

The glassware used in molecular and microbiological work, were sterilized in hot air oven (DTC-96, Expo Hi-Tech, Mumbai) at 180°C for two hours. The media, pipette tips, etc. were sterilized by autoclaving at 121°C for 15 minutes.

3.7. Microbiological Parameters

3.7.1. Bacterial diversity study in biofloccs

For microbiological study, the samples were collected from the respective treatment tanks fortnightly during morning hours of the day between 8.00 and 10.00 am. The biofloc suspensions was taken in the Imhoff cone and allowed to settle for 30 minutes. The settled biofloc suspension were transferred aseptically into sterile Uricol (Hi-Media Laboratories Limited, Mumbai) bottles, and stored in refrigerated condition**.**

3.7.2. Enumeration of total heterotrophic bacteria

Total heterotrophic bacteria were enumerated on nutrient agar medium from all the treatment tanks. 10 ml of the biofloc sample was transferred aseptically to the 90 ml physiological saline which gives 10 times dilution of the sample, i.e. 10^{-1} dilution for further dilution, 1ml from 10^{-1} was mixed with 9 ml saline (10^{-2}) and so on to get the appropriate dilution for plating. Three dilutions were selected for plating in triplicate. 0.1 ml of each of the dilutions was inoculated on the surface of the respective pre-set agar plates. By using glass spreader the inoculum was uniformly spreaded well on the surface of the agar medium. After 30 minutes, the plates were incubated at 37°C for 48 hrs. After 48 hours of incubation, the colonies were enumerated using a colony counter. The average colony counts of triplicate plates were taken for the total heterotrophic bacterial count and expressed as colony forming unit per ml (CFU ml^{-1}).

3.7.3. DNA extraction from biofloc

1 ml of biofloc suspension was kept in Hi-Bead tubes (Hi-Media Laboratories Limited, Mumbai) and the lysozyme-SDS based phenol-chloroform method of extraction was followed with slight modifications; 500 µl of lysis 1 solution (0.15 *M* NaCl, 0.1 *M* EDTA; pH 8; 15 mg lysozyme/l) was added to the

bead tube and mixed by horizontal vortexing for 2 minutes. It was then incubated at 37°C for 1 hour in a water bath. After that, 500 µl of lysis 2 (0.1 *M* NaCl, 0.5 *M* Tris-HCl; pH 8; 12% SDS) solution was added to it and incubated again for 1-2 hours at 60°C in a water bath. The suspension was then centrifuged at 8000 *g* for 10 minutes in a Spinwin (Tarson, India) centrifuge and 1 ml of supernatant was recovered in separate vials. To this supernatant, 1 ml of saturated phenol (pH 8) was added, mixed and centrifuged at 8000 *g* for 8 minutes. The aqueous layer was recovered and an equal volume of chloroform was added, mixed and centrifuged for 5-6 minutes in Spinwin. After centrifugation, the aqueous layer was again transferred to separate tubes, and 2 volumes of absolute ethanol were added and centrifuged for 30 minutes at 4°C at 8000 *g* in a microprocessor-based high speed research refrigerated centrifuge (Eltek, India). The supernatant was decanted and pellets were washed with 500 µl of 70% ethanol and centrifuged at 8000 *g* for 15 minutes using the centrifuge. Finally, the vials were decanted again and kept in a dry bath (SLM-DB-120, Bangalore Genei, India) at 60°C for some time to evaporate the remaining ethanol. The DNA was then re-suspended in 50 µl of nuclease-free water and stored at -20°C.

3.7.4. Genomic DNA confirmation by Agarose Gel Electrophoresis

The elute was checked for the presence of genomic DNA using 1.5% agarose gel pre-stained with ethedium bromide (0.5 µg ml^{-1}). 1.0 µl of 6X loading dye was mixed with 10.0 µl of genomic DNA and loaded into the wells of the gel. 1.0 µl of loading dye was mixed with 5.0 µl 1kb DNA molecular weight marker and loaded into the wells. Electrophoresis was carried out in 1x TAE buffer at 70 volts for about 60 minutes. The gel was visualised and photographed using Bio-Rad Universal Hood II Gel Imager (Bio-Rad Laboratories, USA).

3.7.5. PCR amplification using universal primers

PCR amplification of bacterial 16S rRNA genes was performed using universal primers (Eurofins Genomics India Pvt. Ltd., Bangalore) F968 and R1378

attached with GC clamp. The primer sequences, used in this study are given in Table 2 (Brons *et al.,* 2008).

Table 2. Primer sequences used in the study

Sr. No	Primer[a]	Sequence (5′→3′)
1.	With GC clamp F968⊥GC	CGCCCGGGGCGCGCCCCGGGCGGGGCGGG GGCACGGGGGG -AACGCGAAGAACCTTAC[b]
2.	R1378	CGGTGTGTACAAGGCCCGGGAACG
3.	Without GC clamp F968	AACGCGAAGAACCTTAC

*a*F, forward primer; R, reverse primer; *b*5′ GC clamp (up to hyphen).

The components of PCR reaction are described in Table 3 and the reaction was carried out in a Quanta Biotech Thermo-cycler QB-96 (Quanta Biotech Ltd., UK).

Table 3. Components of PCR reaction

Sr. No	Reagents	Volume (50 µl)
1.	Master mix	25 µl
2.	Forward primer (100 picomole)	0.4 µl
3.	Reverse primer(100 picomole)	0.4 µl
4.	Template	3 µl
5.	Nuclease free water	21.2 µl

The PCR consisted of 30 cycles each of initial denaturation at 95°C for 5 minutes, denaturation at 95^{o}C for 30 seconds, annealing at 61.3^{o}C for 30 seconds and extension at 72^{o}C for 1 minute, and a final extension for 15 minutes. The PCR products were examined by electrophoresis on 1% agarose gel pre-stained with ethedium bromide and visualized in Bio-Rad Universal Hood II Gel Imager (Bio-Rad Laboratories, USA).

3.7.6. DGGE for PCR products

Denaturing gradient gel electrophoresis (DGGE) was performed with the DCode Universal Mutation Detection System (Bio-Rad Laboratories, USA) as per the manufacturer's instructions. PCR samples (10µl about 500 ng), were loaded onto 1-mm thick 6% (wt/vol) polyacrylamide: bisacrylamide gels in 1X TAE buffer (40 *mM* Tris-acetate, 1.0 *mM* EDTA, pH 7.4). The polyacrylamide gels were made with denaturing gradients ranging from 40 to 55% (where the 100% denaturant contained 7 *M* urea and 40% (v/v) formamide (Bangalore Genei, Bangalore). The gels were run for 14 h at 60 V and 60°C.

3.7.7. Silver staining of DGGE gel

The method described by Sanguinetti *et al.* (1994) was modified and followed to stain the DGGE gels. The stock solutions were prepared as follows:

Fixing solution: 3 g of benzene sulfonic acid was added into the 100 ml of 24% ethanol and stored in amber colour bottle.

Staining solution: 0.35 g of benzene sulfonic acid and 1 g silver nitrate was dissolved in double distilled water and made up to 100 ml.

Developing solution: 32.25 g of anhydrous sodium carbonate was dissolved in double distilled water and made up to 250 ml.

All the working solutions were prepared from the stock solutions. The gels were first fixed with fixing solution (48 ml ethanol, 100 ml stock solution and 102 ml of double distilled water) for 30-45 minutes with gentle shaking. Then, the gels were stained with staining solution (50 ml stock solution and 200 ml double distilled water) for 30-45 minutes with gentle shaking in dark place. The gels were washed for 1 minutes with double distilled water and the developer solution {(50 ml stock solution, 200 ml double distilled water, 250 µl sodium thiosulphate (20 mg/ml) and 250 µl formaldehyde} was poured into the tray. The first precipitate formed was quickly aspirated and the rest of the developer was added. After 5-10

minutes, the gels were soaked in 3% acetic acid for 5-10 minutes to stop further development. The gel images were recorded with Bio-Rad Universal Hood II Gel Imager (Bio-Rad Laboratories, USA) with white light settings.

3.7.8. Sequencing of excised DGGE bands

Distinct bands in all the lanes were marked, and bands which were common in a number of lanes were considered similar and only one band each from the similar positions of the polyacrylamide gel was eluted for sequencing. The prominent bands from the DGGE gels were excised with sterile cover slips and soaked overnight in 25 µl of nuclease-free water after little crushing. The PCR re-amplification was carried out using the same set of primers, but without GC clamp in a Quanta Biotech Thermo-cycler QB-96 (Quanta Biotech Ltd., UK). The components of re-PCR reaction are described in Table 4.

Table 4. Components of re-PCR reaction

Sr. No	Reagents	Volume (30 µl)
1.	Master mix	20 µl
2.	Forward primer (100 picomole)	0.3 µl
3.	Reverse primer(100 picomole)	0.3 µl
4.	Template	1 µl
5.	Nuclease free water	8.4 µl

The re-PCR consisted of 30 cycles each of initial denaturation at 95°C for 3 minutes, denaturation at 95^{o}C for 1 minute, annealing at 60.8^{o}C for 30 seconds and extension at 72^{o}C for 1 minute, and a final extension for 15 minutes. The re-amplified PCR products were confirmed through electrophoresis on 1.6% agarose gel pre-stained with ethedium bromide and visualized in a Bio-Rad Universal Hood II Gel Imager (Bio-Rad Laboratories, USA). The re-PCR products were stored at -80°C and directly send for sequencing to Banglore Genei, India.

The partial bacterial 16S rRNA gene sequences were subjected to BLAST (www.ncbi.nlm.nih.gov/BLAST/) search on the GenBank nucleotide database NCBI (National Centre for Biotechnology Information) to identify the sequences with higher similarity.

3.8. Water quality parameters

Water samples were collected at fortnightly interval from the experimental tanks after stocking during morning hours between 8.00 and 9.00 am for a period of three months. Temperature and pH was measured in the experimental unit itself. Estimation of dissolved oxygen and biochemical oxygen demand, water samples were collected separately in respective bottles following the APHA (2005) guidelines for sample collection and preservation. For analyse of other water quality parameters, water samples were collected in 1L polyethylene bottles and stored at 4°C. Water quality parameters were estimated in the laboratory using standard procedures.

3.8.1. Temperature

Water temperature of the tanks was recorded using a digital Multi thermometer (H-9283, Shenzhen Vicimeter Technology Co. Ltd, China).

3.8.2. Total alkalinity

The total alkalinity of water was estimated titrimetrically using phenolphthalein and methyl orange as indicators. Both carbonate and bicarbonate alkalinities were measured following the standard methods (APHA, 2005).

3.8.3. pH

The pH was measured using a portable digital pH meter (Eutech Instruments, Malaysia).

3.8.4. Hardness

The hardness of water was estimated titrimetrically using Eriochrome Black T as indicators by following the standard methods (APHA, 2005).

3.8.5. Dissolved oxygen

The dissolved oxygen content of the water sample was estimated by Winkler's titrimetric method (APHA, 2005). The samples were collected in a 300 ml BOD bottle without air bubbles. The dissolved oxygen was fixed with 1 ml $MnSO_4$ and 1 ml alkali iodide-azide reagent at the experimental site. The samples were brought to the laboratory and 1 ml of conc. sulphuric acid was added and thoroughly mixed by inverting the bottles. After solution became clear, 100 ml of sample was taken for titration against 0.01*N* Sodium thiosulfate in the presence of starch indicator. The sample was titrated till end point disappearance of blue colour. Dissolved oxygen content of the sample was calculated from the titre value and is expressed as mg L^{-1}

3.8.6. Total Ammonia-N (TAN)

Ammonia-N in the water samples was estimated colorimetrically using Phenate method (APHA, 2005). 1 ml phenol solution, 1 ml sodium nitroprusside solution and 2.5 ml oxidizing solution (alkaline citrate + sodium hypochlorite) were added to 25 ml filtered sample in an Erlenmeyer flask. The samples were then covered with aluminium foil and incubated at room temperature in subdued light. After 2 hours, the Indo-phenol blue colour developed was measured with a double-beam spectrophotometer (Thermospectronic, UV 1, UK) at 640 nm. Blank and standards were also measured in the same way.

3.8.7. Nitrite-N

Nitrite-nitrogen was determined using the Colorimetric Method (APHA, 2005). 25 mL of filtered water sample were taken in to an Erlenmeyer flask and 1 mL of colour reagent (100 ml of 85% phosphoric acid, 10 g sulphanilamide and 1 g N-(1-naphthyl)-ethylenediamine di-hydrochloride (NEDD)made up to 1L with distilled water) was added into it. The nitrite produces a red azo-dye by diazotizing with sulphanilamide and coupling with NEDD. After 15 minutes, the absorbance was measured at 543 nm using a double-beam spectrophotometer (Thermospectronic, UV 1, UK). Blank and standards were also measured in the same way.

3.8.8. Nitrate-N

Nitrate-nitrogen was determined using cadmium reduction method (APHA, 2005). Nitrate is reduced almost quantitatively to nitrite in the presence of cadmium. This method employed the commercially available cadmium granules treated with copper sulphate and packed in a glass column. 25.0 mL of sample was mixed with 75 ml NH_4Cl-EDTA solution. The mixed sample was poured into column and collected reduced sample at a rate of 7 to 10 ml/min. The first 25 mL of sample was discarded and rest of the sample were collected in the original sample flask. After the reduction the procedure in 3.8.7. was followed for the measurement

3.8.9. Total suspended solids (TSS)

A known volume of well-mixed sample was filtered through a weighed standard glass-fiber filter and the residue retained on the filter was dried in hot air oven (Newtronic, Mumbai) to a constant weight at 103 to 105°C (APHA, 2005). TSS is expressed in mg L^{-1}.

3.8.10. Volatile suspended solids (VSS)

A known weight of dried sample was ignited in the muffle furnace (AI-7981, Expo Hi-Tech, Mumbai) to a constant weight at 550°C (APHA, 2005). The weight difference of the sample before and after ignition was taken for calculation of volatile suspended solids and it's expressed in mg L^{-1}.

3.8.11. Electrical conductivity

The electrical conductivity of water was measured using a digital conductivity meter (Eutech Instruments, Thermo Scientific, Singapore) and is expressed as mS cm^{-1}.

3.8.12. Biochemical oxygen demand

Dissolved oxygen was measured by Winkler's method before incubation and after incubation at 20°C for 5 days in closed BOD bottles without seeding. BOD was calculated by subtracting the final DO from initial DO. BOD of the sample is expressed as mg L^{-1}

$$BOD_5 \text{ (mg } L^{-1}) = [(\text{Initial DO - Final DO}) \times V_B]/ V_S$$

Where,

V_B= volume of BOD bottle,

V_S= volume of sample taken for titration

3.8.13. Floc volume

Floc volume was measured by using the Imhoff Cone (Merck specialties, India). The biofloc suspension was taken in the Imhoff Cone and allowed to settle for 30 minutes. After that, volume occupied by the settled biofloc was measured in ml.

3.8.14. Carbon nitrogen ratio (C/N)

The carbon nitrogen ratio in water samples was estimated using CHNS Vario Micro Cube (Elementar Analysysteme GmbH, Germany). The water samples (20 µl) were filled in pre-weighed aluminium vials and weighed in microgram level using Mettler Toledo MX/UMX balance (Mettler-Toledo Laboratory and Weighing Technologies, Switzerland). The vials were sealed properly without air introduced into the auto analyser.

3.9. Biological Parameters

The samples were collected from the respective treatment tanks at fortnightly basis during morning hours of the day between 8.00 and 10.00 am following standard sampling procedures.

3.9.1. Chlorophyll estimation

The algae from the water samples from the treatment tanks were concentrated using a syringe filter assembly from Millipore (India) Pvt. Ltd., Bangalore, India. Cellulose acetate filters of 0.45 µm diameter were used to filter 100 ml of water. After filtration, the filters with plankton concentrate were preserved in 90% acetone in amber coloured bottles. The pigments were extracted from the plankton concentrate with aqueous acetone and the absorbance of the extract was estimated by using with a double-beam spectrophotometer (Thermospectronic, UV 1, UK) at 750, 664, 647, and 630 nm for the estimation of chlorophyll-a, following trichromatic method (APHA, 2005). The concentration of chl a is expressed in µg L^{-1}.

3.9.2. Plankton analysis

The plankton samples were collected in duplicate and concentrated to 50 ml by filtering the water using bolting silk cloth (no. 25) from the respective treatment tanks. The collected plankton samples were preserved in 5% formalin

for further analyses (Phillpose, 1959; Pennak, 1978). Organisms were identified using keys and monographs given by Edmondson (1992), Lund and Lund (1998), Desikacharya (1959), Graham *et al*. (2008), Prescott (1969), APHA (2005) and Adoni (1975). The counting of plankton was carried out using a Sedgwick-Rafter counting cell, adopting the procedure outlined by Welch (1948). The averages of three samples were taken into consideration and the results are given in terms of cells per litre. The photographs of all the major planktons were taken using a camera (JVC Color Video Camera, TK-C1481BEG, Thailand) attached to a Hund microscope (H600L, HundWetzlar, Germany). The morphometric features of the organisms were measured using the Bio-wizard software.

3.10. Growth Parameters

The sampling was performed at fortnightly interval for 90 days to assess the body weight of the shrimps. Shrimps were starved overnight before taking the weight. Twenty percent of the test animals were randomly weighed for all the treatments. The weight was taken in an electronic balance. Weight of feed offered recorded accurately.

3.10.1. Total weight gain

Total weight gain was calculated using the following formula

Total weight gain (wet weight, g) = final weight (g) – initial weight(g)

3.10.2. Average body weight

The Average body weight was calculated using the following formula

$$\text{Average body weight (g)} = \frac{\text{total weight (g)}}{\text{total Number of animals}}$$

3.10.3. Growth rate

The growth rate was calculated using the following formula

$$\text{Growth rate (g day}^{-1}\text{)} = \frac{\text{final weight (g)} - \text{initial weight (g)}}{\text{number of days}}$$

3.10.4. Specific growth rate (SGR)

The specific growth rate was calculated by the following formula

$$\text{SGR (\%)} = \frac{\ln \text{final weight} - \ln \text{initial weight}}{\text{number of days}} \times 100$$

3.10.5. Feed conversion ratio (FCR)

The feed conversion ratio was calculated by the following formula

$$\text{FCR} = \frac{\text{feed consumed (dry weight) (g)}}{\text{body weight gain (wet weight) (g)}}$$

3.10.6. Feed efficiency ratio (FCR)

The feed efficiency ratio was calculated by the following formula

$$\text{FER} = \frac{1}{\text{FCR}}$$

3.10.7. Protein efficiency ratio (PER)

Protein efficiency ratio was calculated by the following formula

net weight gain (wet weight) (g)

$$PER = \frac{\text{protein consumed (g)}}{\text{protein consumed (g)}}$$

3.10.8. Survival rate

At the end of the experiment, all the experimental tanks were dewatered and the number of the experimental animals in each tank was counted and the survival rate (%) was calculated by the following formula

$$\text{Survival (\%)} = \frac{\text{total number of animals harvested}}{\text{total number of animals stocked}} \times 100$$

3.11. Nutritional Composition

3.11.1. Proximate Analysis of Biofloc, Feed, Carbon Source and Tissues

The shrimps and bioflocs were collected from the experimental tank at the end of the experiment and the animal biomass of the respective tanks was noted down. The samples were then stored at -20°C for further analysis. It was done by the prescribed method (AOAC, 2005). Similarly, the proximate composition of all the experimental diets was estimated.

3.11.1.1. Moisture

The moisture content of the diets and animal tissue was determined by taking a known weight of the sample in the Petri dish and drying it in a hot air oven (Newtronic, Mumbai) at 105°C till a constant weight was achieved. The difference in weight of the sample gave the moisture content, which was calculated by using the following formula:

$$\text{Moisture (\%)} = \frac{\text{wet weight of sample (g)} - \text{dried weight of sample (g)}}{\text{wet weight of sample (g)}} \times 100$$

3.11.1.2. Crude Protein (CP)

The crude protein was calculated by multiplying the total nitrogen with a factor 6.25. The total nitrogen was estimated by using automated nitrogen analyser (KEL Plus – Classic DX VA, Pelican Equipments, India). For digestion, 0.5 g of sample was placed in a digestion flask and added 5 g of catalyst mixture followed by 10 ml concentrated sulphuric acid. The digestion was carried out in the Kjeldhal automatic sample digestion system at 450°C for 2 hours. The digested samples were distillated in the distillation unit using sodium hydroxide. The liberated ammonia was trapped in boric acid and titrated against 0.01*N* HCl by using double indicator (bromocresol green: methyl red). The total nitrogen was calculated from the titre value. The crude protein content was calculated by the following formula:

Crude Protein (%) = nitrogen (%) X 6.25

3.11.1.3. Ether Extract (EE)

The ether extract was estimated by automatic fat extraction system (SOCS PLUS-SCS 08 AS, Pelican Equipments, India) using petroleum ether (Boiling point 60-80ºC) as the solvent. Sample (1.0 g) was placed in a thimble, and then it was kept in the pre-weighed glass beaker which contained petroleum ether. The extraction was continued for 45 minutes. Beakers were fitted with the heating mantle and ran as per the instrument guidelines. After extraction, remaining ether was evaporated and the beaker was dried at 120°C (Newtronic, Mumbai). The dried beakers were weighed and the weight was recorded. The weight difference in the beaker before and after extraction gives the weight of ether extract present in the sample. The ether extract is expressed as weight in percentage.

$$\text{Ether Extract (\%)} = \frac{\text{weight of the ether extract (g)}}{\text{weight of the sample (g)}} \times 100$$

3.11.1.4. Crude Fiber (CF)

The crude fiber was estimated by using automatic fiber analysis system (FIBROTRON-FRB 8, Tulin Equipments, India). The fat and moisture free sample (1.0 g) were placed in a pre-weighed crucible and fitted into the instrument. The samples were refluxed with 100 ml of 1.25% sulphuric acid followed by 1.25% NaOH. Each washing continued for 45 minutes. After completions of both the washes, the crucibles were dried in the hot air oven at 105° C for 3-4 hours. The crucibles were cooled in the desiccator and weighed. The dried samples were ignited at 550°C for 4-5 hours, and then the crucibles were cooled and weighed. The weight difference in the samples before and after ignition gives the crude fiber of the sample and it is expressed as weight in percentage.

$$\text{Crude Fiber (\%)} = \frac{\text{wt. sample before ashing} - \text{wt of sample after ashing}}{\text{weight of sample taken}} \times 100$$

3.11.1.5. Total Ash

The Total ash content was estimated by taking a known weight of sample in silica crucible and placing it in a muffle furnace (AI-7981, Expo Hi-Tech, Mumbai) at 550°C for 6 hours. The calculation was done as follows:

$$\text{Total Ash (\%)} = \frac{\text{weight of ash}}{\text{weight of sample}} \times 100$$

3.11.1.6. Nitrogen Free Extract (NFE)

The nitrogen free extract was calculated by subtracting the percentage of other nutrients (moisture, ash, crude protein, ether extract and crude fibre) from 100 (AOAC, 2005).

NFE (%) = 100 – (Moisture% + Crude protein% – Ether extract% – Total Ash% – Crude fiber %)

3.11.1.7. Total Carbohydrate (TC)

The total carbohydrate of fish tissues was calculated by the formula given by Hasting (1969).

TC (%) = 100 – (Moisture% + Crude protein% + Ether extract% + Total Ash %)

3.11.1.8. Gross energy (GE)

Gross energy content of biofloc sample was determined by using a bomb calorimeter (Parr Calorimetric Thermometer-6772, Parr Instruments Company, USA). Briefly, energy of the dried material was determined by placing a weighed sample in the ignition cup of the bomb. Oxygen was filled at 25 psi and the bomb immersed in 2 L of water. The sample was ignited and the temperature increase of the water recorded. Gross energy is calculated using a boric acid calibration standard and expressed as kcal g^{-1} (kJ kg^{-1}) of dry matter.

3.12.2. Mineral composition of biofloc

3.12.2.1. Total Phosphorus

The biofloc samples were digested using Supra pure concentrated acids (Merck) in a microwave-based digestion system (Microwave 3000, Anton Parr, USA). Three replicates of about 0.250 g of biofloc samples were taken in the microwave digestion vessels, to which 5 ml of conc. HNO_3, 2 ml of conc. HCl and 1 ml of conc. HF were added. The vessels were capped and heated in the microwave unit at 1200 W to a temperature of 190°C for 30 minutes at a pressure of 25 bars. The digested samples were diluted to 50 ml and were used for the analysis of total phosphorus.

All the glassware, used for the analysis, was treated with chromic acid wash solution overnight and washed with distilled water. Aliquots of 5 ml each of the digested samples were taken in a 50 ml volumetric flask and the pH was adjusted to 5 using 5 *N* NaOH and *p*-nitrophenol as the indicator. The standard colorimetric ascorbic acid method as mentioned in APHA (2005) was used for the estimation. The colour development was measured after 10 minutes, but before 30 minutes, using a double-beam spectrophotometer (Thermospectronic, UV 1, UK) set at 880 nm. A blank and standards were run simultaneously. The absorbance values were plotted on a standard curve to obtain the concentration of phosphorus in the samples.

3.12.2.2. Sodium and Potassium

The biofloc samples were digested the same procedure as that for total phosphorus. Sodium, and potassium in the samples were then estimated using a flame photometer (Elico CL 378, India). The samples were loaded into the aspirator where it got aspirated into the burner and dispersed to the flame as a fine spray.

3.12.2.3. Calcium, Magnesium, Zinc, Copper and Iron

The biofloc samples were digested the same procedure as that for total phosphorus. Analysis of the five elements (Ca, Mg, Zn, Cu and Fe) by atomic absorption spectrophotometer (AAnalyst 800, Perkin Elmer, USA) using flame atomization.

3.13. Statistical analysis

All statistical analysis was performed using SAS v9.3 for Windows (Cary, North Carolina, USA). Water quality parameters were compared by two-way repeated measures ANOVA with treatment as main factor and sampling date as repeated measures factor. One-way analysis of variance (ANOVA) was performed to examine difference of growth parameters, proximate composition

and nutritional quality of biofloc among treatments and control. Correlation matrix was also used in the present study to understand the significant correlations between biofloc proximate composition and growth parameters of the treatments. The analyses were run at 5% significance level.

4. RESULTS

4.1. Bacterial diversity

To investigate the bacterial diversity of bioflocs, the genomic DNA extracted from the bioflocs were subjected to denaturing gradient gel electrophoresis (DGGE) with PCR amplified 16S rRNA gene using universal DGGE primers, F968 and R1378 (Fig. 2). The gel images obtained after DGGE are given in figures 4 to 7. The excised DGGE bands were further subjected to re-PCR and the amplification was confirmed by running agarose gel electrophoresis (Fig. 3). The PCR products were then purified and sequenced; fourteen different sequences were obtained from BFT_S sediment, thirteen from BFT_T, sixteen from BFT_W and five from control. Comparison of the 16S rRNA gene sequence, derived from the DGGE bands with those of GenBank database revealed the identity of the organisms (Table 5). The dominant bacteria in biofloc treatment (BFT_S) were *Saccharophagus degradans, Blattabacterium* sp*., Shewanella sediminis, Shewanella woodyi, Simiduia agarivorans, Ruegeria* sp*., Phaeobacter gallaeciensis, Roseobacter litoralis, Photobacterium profundum, Hyphomonas neptunium, Mycoplasma pulmonis, Coprococcus* sp*, Chitinophaga pinensis* and *Treponema primitia*. The distinct bacterial species observed in biofloc treatenemt (BFT_T) were *Shewanella woodyi, Pseudomonas mendocina, Shewanella denitrificans, Roseobacter litoralis, Rhodanobacter* sp*., Ruegeria* sp*., Phaeobacter gallaeciensis, Candidatus Blochmannia chromaiodes* str. *640, Clostridium pasteurianum, Hyphomonas neptunium, Coprococcus* sp*, Chitinophaga pinensis, Candidatus Arthromitus* sp*.* The dominant bacteria in biofloc treatment (BFT_W) were *Xanthomonas campestris, Roseobacter litoralis, Glaciecola psychrophila, Shewanella denitrificans, Rhodanobacter* sp*., Ruegeria* sp*., Phaeobacter gallaeciensis, Octadecabacter antarcticus, Candidatus Blochmannia chromaiodes* str*. 640, Shewanella piezotolerans, Ketogulonigenium vulgarum, Hyphomonas neptunium, Mycoplasma pulmonis, Pantoea ananatis, Chitinophaga pinensis, Candidatus Arthromitus* sp*.* Whereas *Pseudomonas mendocina, Roseobacter litoralis, Thiocystis violascens, Clostridium*

pasteurianum and *Treponema primitia* were the bacteria observed in the control.

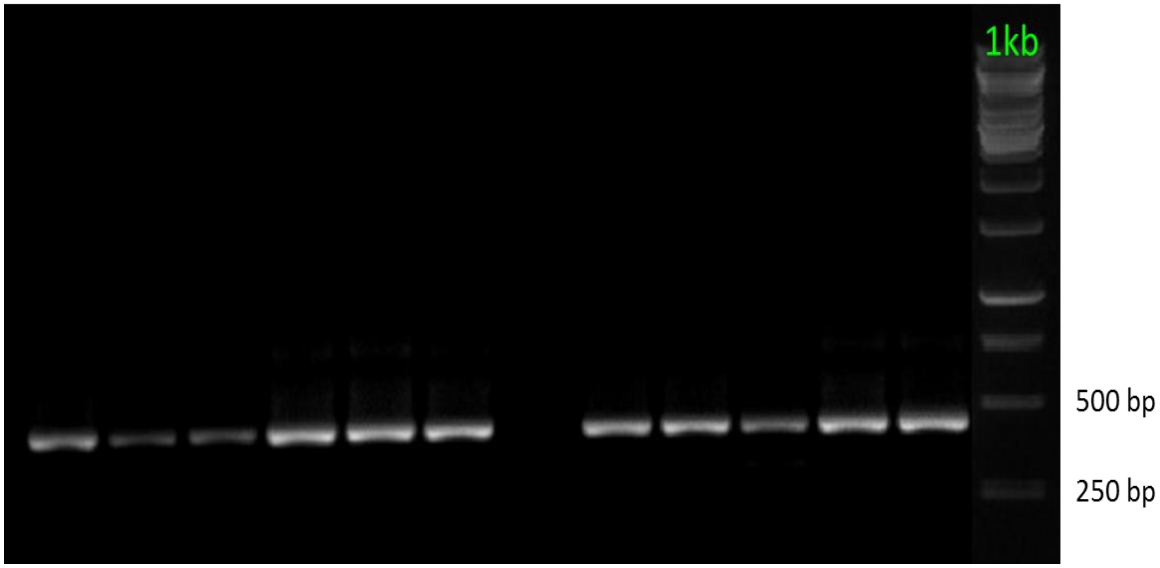

Fig.2. Agarose gel electrophoresis picture of 410 bp DGGE amplicon obtained using F968 and R1378

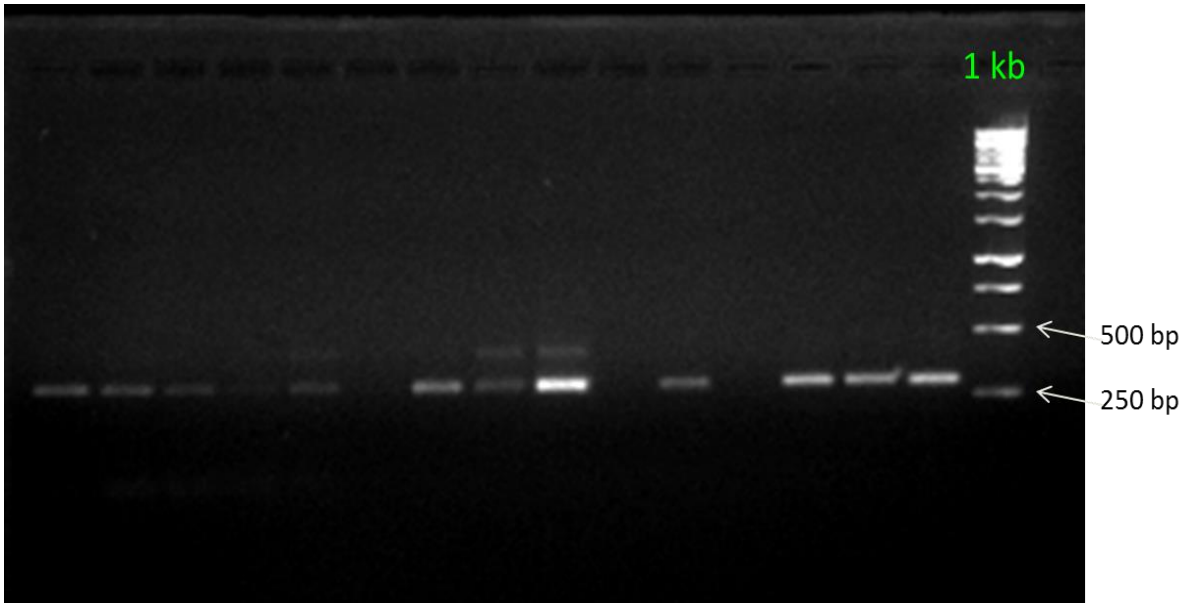

Fig. 3. Agarose gel electrophoresis picture of 300bp amplicon obtained after re-PCR of excised DGGE bands

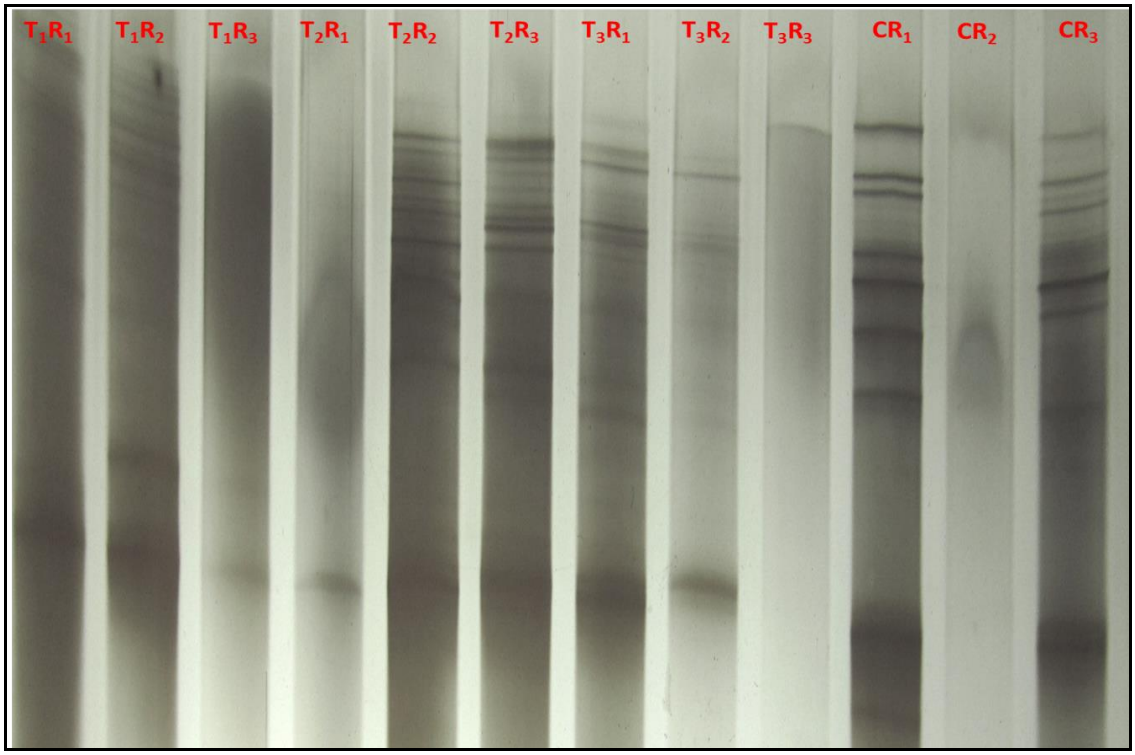

Fig. 4. DGGE gel showing the microbial diversity on 30th day of the experiment (9 different bands obtained from this gel)

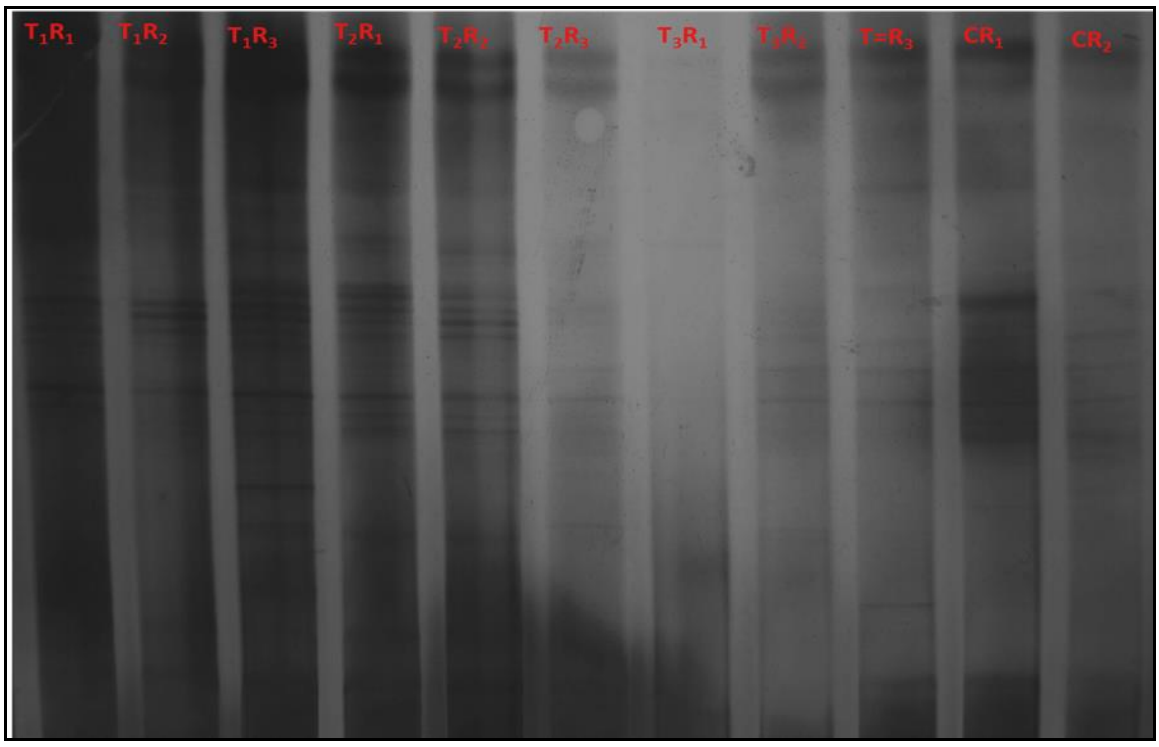

Fig. 5. DGGE gel showing the microbial diversity in biofloc on 60th day of the experiment (9 different bands obtained from this gel)

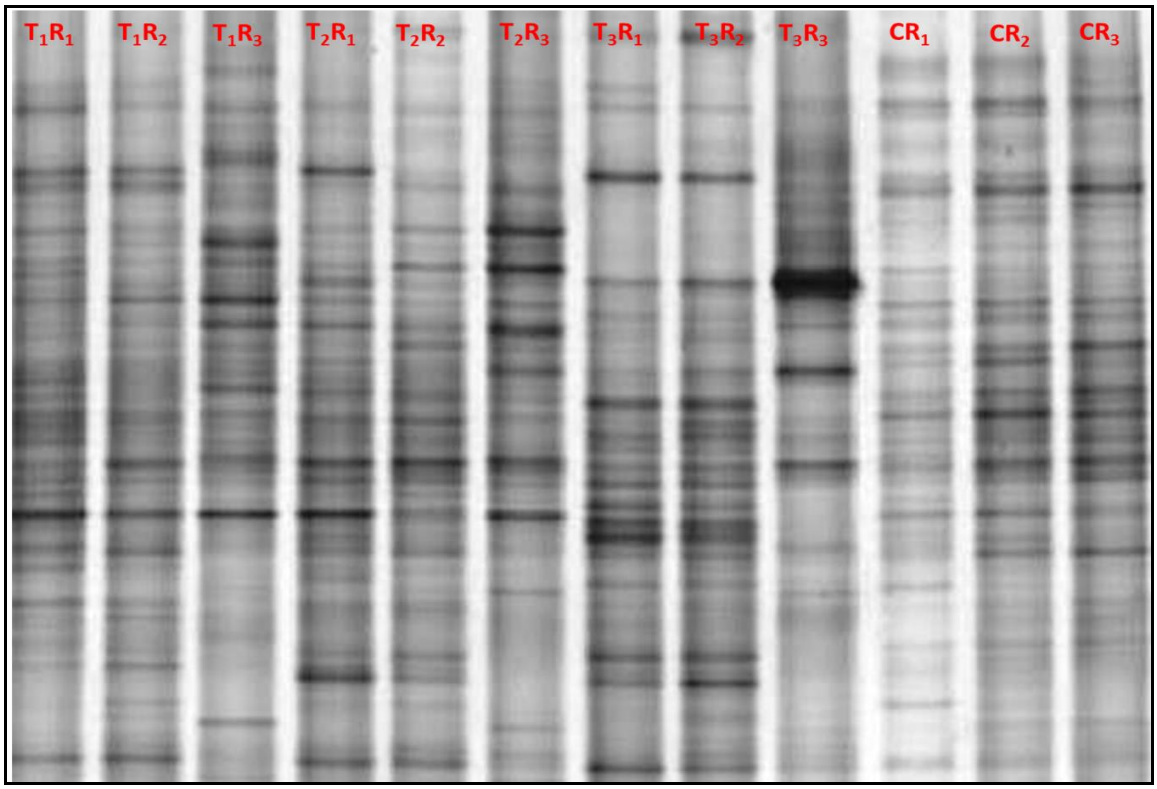

Fig. 6. DGGE gel showing the microbial diversity on 90th day of the experiment (35 different bands obtained from this gel)

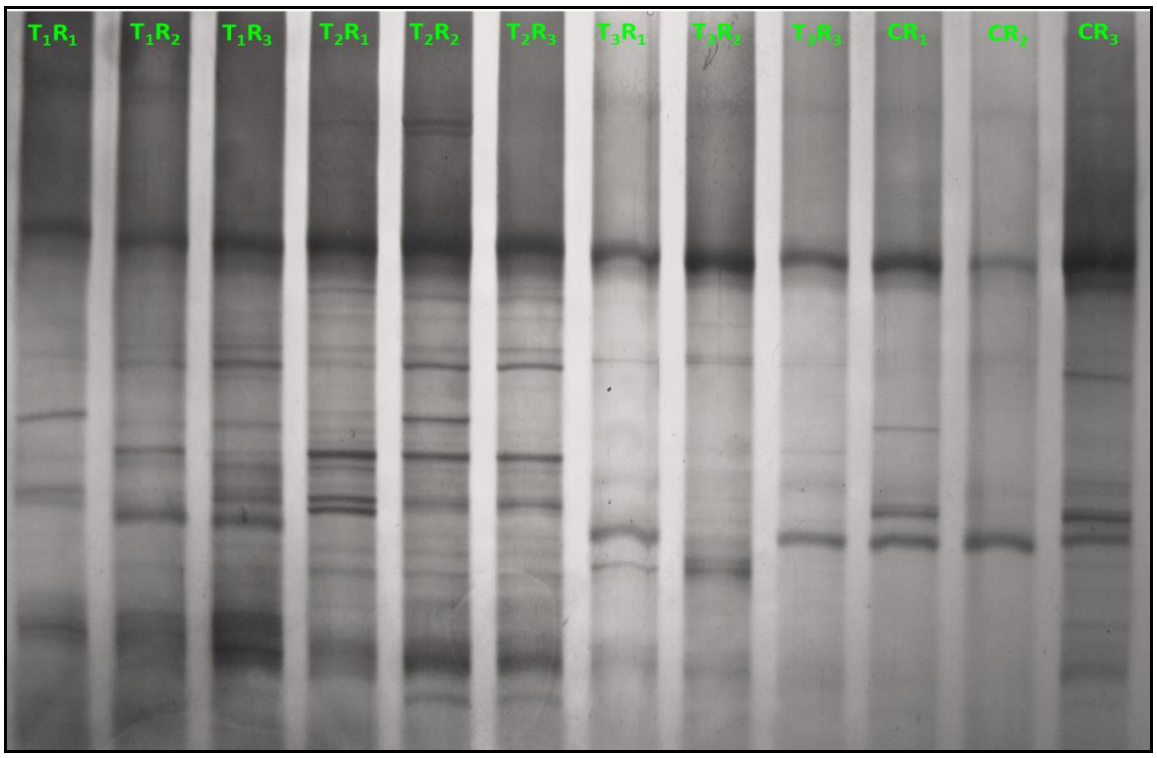

Fig. 7. DGGE gel showing the microbial diversity on 120th day of the experiment (16 different bands obtained from this gel)

Table 5. Bacteria identified from different biofloc treatments and control

Sl. No	Closest phylogenetic neighbour	Similarity	BFT_S	BFT_T	BFT_W	C
1.	*Saccharophagus degradans*	96%	+	-	-	-
2.	*Blattabacterium sp.*	87%	+	-	-	-
3.	*Shewanella sediminis*	79%	+	-	-	-
4.	*Shewanella woodyi*	92%	+	+	-	-
5.	*Xanthomonas campestris*	71%	-	-	+	-
6.	*Pseudomonas mendocina*	88%	-	+	-	+
7.	*Glaciecola psychrophila*	86%	-	-	+	-
8.	*Shewanella denitrificans*	82%	-	+	+	-
9.	*Rhodanobacter sp.*	80%	-	+	+	-
10.	*Simiduia agarivorans*	78%	+	-	-	-
11.	*Ruegeria sp.*	100%	+	+	+	-
12.	*Phaeobacter gallaeciensis*	98%	+	+	+	-
13.	*Octadecabacter antarcticus*	85%	-	-	+	-
14.	*Roseobacter litoralis*	88%	+	+	+	+
15.	*Photobacterium profundum*	87%	+	-	-	-
16.	*Thiocystis violascens*	84%	-	-	-	+
17.	*Candidatus Blochmannia chromaiodes str. 640*	84%	-	+	+	-
18.	*Shewanella piezotolerans*	79%	-	-	+	-
19.	*Clostridium pasteurianum*	100%	-	+	-	+
20.	*Ketogulonigenium vulgarum*	72%	-	-	+	-
21.	*Hyphomonas neptunium*	68%	+	+	+	-
22.	*Mycoplasma pulmonis*	100%	+	-	+	-
23.	*Pantoea ananatis*	93%	-	-	+	-
24.	*Coprococcus sp*	89%	+	+	-	-
25.	*Chitinophaga pinensis*	93%	+	+	+	
26.	*Treponema primitia*	92%	+	-	-	+
27.	*Candidatus Arthromitus sp.*	85%	-	+	+	-

[(**+**) presence and (**-**) absence of organism]

4.2. Water quality Parameters

4.2.1. Temperature

The water temperature of the different experimental groups ranged from 22.0°C to 24.8°C (Fig. 8) during the experimental period. The lowest water temperature was recorded in the month of January and the highest temperature was recorded in the month of November.

4.2.2. Total alkalinity

The observations presented in table 6 and Fig. 9 shows that there was no significant difference in the total alkalinity among the treatments. The mean total alkalinity ranged from 138.69 to 153.52 mg $CaCO_3$ L^{-1}.

4.2.3. pH

The fluctuation of pH was observed between 6.5 and 8.6 (Fig. 10) during the experiment. The mean pH ranged from 7.6 to 7.8 (Table 6).

4.2.4. Total hardness

The total hardness was found to be 2853 - 5235 mg L^{-1} (Fig. 11) during the experimental period in all the experimental tanks. There was no significant variation among the treatments (Table 6).

4.2.5. Dissolved oxygen

The concentrations of dissolved oxygen in all the experimental tanks were recorded within the range of 4.1 to 8.16 mg L^{-1} (Fig. 12) during the experimental period. Dissolved oxygen showed significant variation ($P<0.05$) among treatments. The BFT_S (5.99 mg L^{-1}) had significantly lower DO than that of BFT_W (Table 6).

4.2.6. Total ammonia-N (TAN)

The concentration of total ammonia nitrogen ranged from 0.04 to 1.1 mg L^{-1} (Fig. 13). The concentration of TAN varied significantly between the

treatments. The highest mean values of 0.78 mg L^{-1} was observed in the control and the lower mean value of 0.24 mg L^{-1} in BFT_W (Table 6).

4.2.7. Nitrite-N

The nitrite-nitrogen concentration fluctuated from 0.16 to 2.38 mg L^{-1} during the experimental period (Fig. 14). The concentration of nitrite-N among the treatments varied significantly (Table 6). The highest mean value of 1.89 mg L^{-1} in control and lower mean value of 0.61 mg L^{-1} was recorded in the treatment BFT_W (Table 6).

4.2.8. Nitrate-N

The nitrate-nitrogen concentration in all the experimental tanks was recorded within the range of 0.74 to 3.67 mg L^{-1} during the experimental period (Fig. 15). The concentration of nitrite-N among the treatments varied significantly (Table 6). However, the mean value of higher concentration of NO_3-N (3.21 mg L^{-1}) was observed in the control and lower concentration 2.09 mg L^{-1} in BFT_W (Table 6).

4.2.9. Total suspended solids

The TSS fluctuation ranged from 42.0 to 733.33 mg L^{-1} in all the experimental tanks (Fig.16). The TSS among treatment varied significantly. The TSS in BFT_W and BFT_T was at par and significantly higher than BFT_S and control (Table 6).

4.2.10. Volatile suspended solids

The VSS concentration fluctuated between 101.33 and 885.33 mg L^{-1} in all the experimental tanks during the study period (Fig. 17). The VSS in BFT_W and BFT_T were at par and were significantly higher than the control (Table 6).

Table 6. Water quality parameters of different experimental groups

Parameters	Control	BFT_S	BFT_T	BFT_W	P value
Temperature (ºC)	23.29^{a}±0.24	23.12^{a}±0.20	23.08^{a}±0.20	23.16^{a}±0.23	0.9140
Alkalinity (mg $CaCO_3$ L^{-1})	138.89^{a}±8.11	153.52^{a}±10.91	150.61^{a}±9.39	138.68^{a}±9.43	0.5763
pH (no unit)	7.8±0.0	7.8±0.0	7.6±0.0	7.7±0.0	0.000
Hardness (mg $CaCO_3$ L^{-1})	4635.85^{a}±84.16	4261.36^{a}±200.10	4456.11^{a}±152.01	4453.97^{a}±109.59	0.3402
DO (mg L^{-1})	6.57^{ab}±0.43	5.99^{b}±0.26	6.70^{ab}±0.36	7.39^{a}±0.44	0.0382
TAN (mg L^{-1})	0.78^{a}±0.10	0.57^{ab}±0.10	0.40^{bc}±0.08	0.24^{c}±0.05	0.0002
Nitrite-N (mg L^{-1})	1.89^{a}±0.14	0.93^{b}±0.09	0.75^{bc}±0.11	0.61^{c}±0.05	<0.0001
Nitrate- N (mg L^{-1})	3.21^{a}±0.14	2.42^{bc}±0.12	2.56^{b}±0.12	2.09^{c}±0.12	<0.0001
TSS (mg L^{-1})	158.5^{b}±29.66	285.08^{b}±36.73	493.50^{a}±69.31	484.94^{a}±65.46	<0.0001
VSS (mg L^{-1})	282.00^{b} ±42.54	427.83^{ab} ±51.93	494.89^{a} ±56.15	526.22^{a} ±59.21	0.0085
EC (mS Cm^{-1})	33.39^{a}±2.09	31.60^{a}±2.47	46.73^{a}±21.23	37.64^{a}±1.82	0.3365
Chlorophyll a (µg L-1)	283.44^{b}±39.41	439.14^{a}±49.13	516.77^{a}±64.30	553.67^{a}±59.99	0.0038
BOD (mg L^{-1})	15.98^{c}±2.42	64.34^{b}±15.16	77.54^{ab}±17.58	108.44^{a}±16.25	0.0003
Floc volume (ml L^{-1})	15.50^{c}±1.75	32.17^{b}±2.88	33.64^{b}±3.25	43.06^{a}±3.60	<0.0001
THB $x10^6$ (cfu ml^{-1})	19.40 ± 139.57^{b}	93.50 ± 107.23^{ab}	131.88^{a}±107.69	148.16^{a}±118.00	0.0425
Plankton $x10^6$ (cells L^{-1})	1.45^{b}±0.08	1.93^{ab}±0.23	2.23^{a}±0.18	2.38^{a}±0.21	0.0041

DO: Dissolved Oxygen; **TAN:** Total Ammonia-N **TSS:** Total Suspended Solids; **VSS:** Volatile Suspended Solids; **EC:** Electrical Conductivity; **BOD:** Biochemical Oxygen Demand; **THB:** Total Heterotrophic Bacteria. Values are means (±SE, n=18) of three replications in six sampling date for treatment and control. Mean values in the same row with different superscript differ significantly (P<0.05).

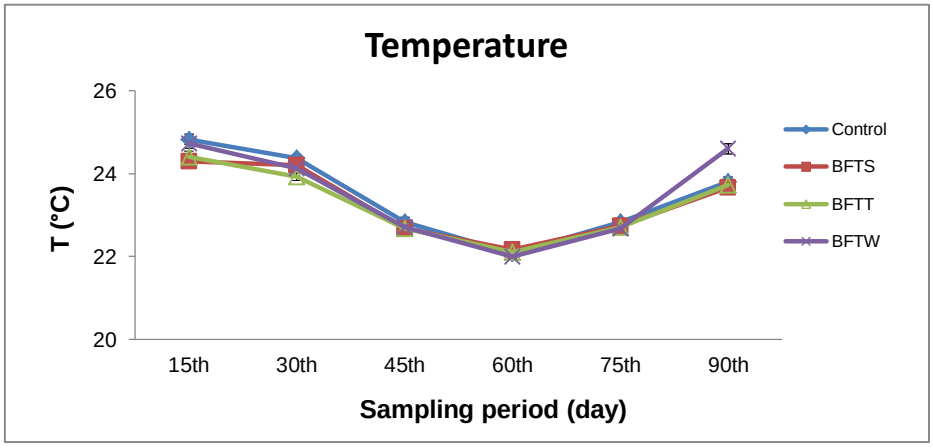

Fig. 8. Temperature (mean±SE) fluctuation in different experimental groups

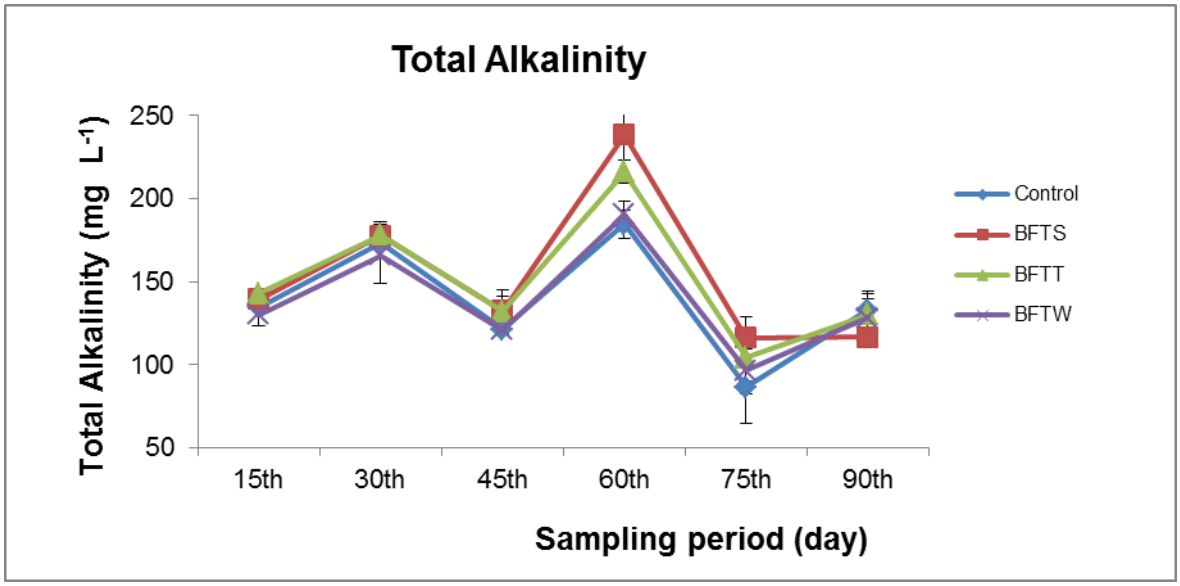

Fig. 9. Total alkalinity (mean±SE) fluctuation in different experimental groups

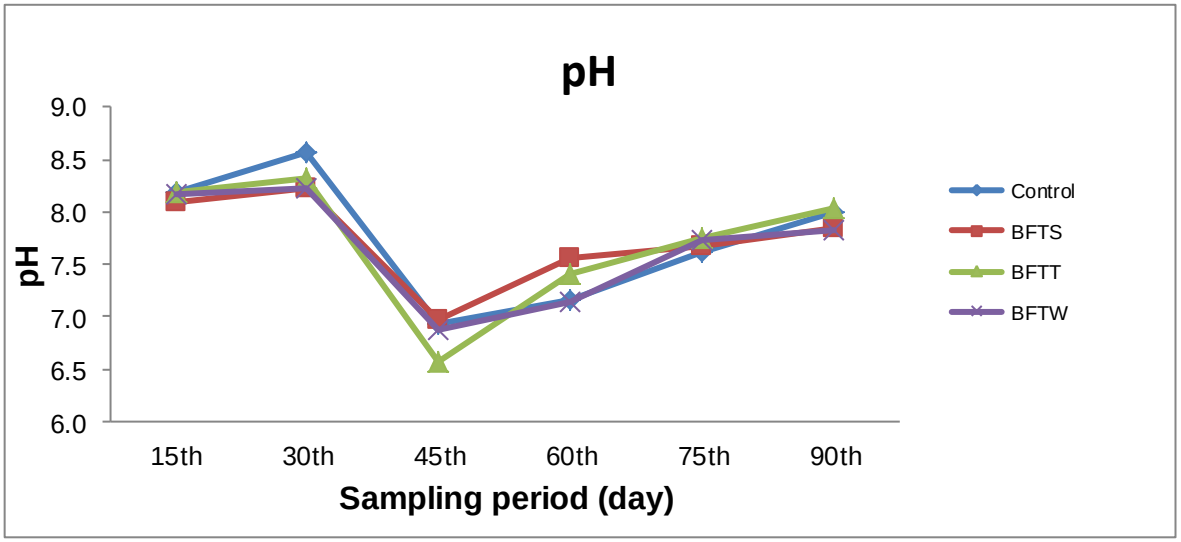

Fig. 10. Variations in pH (mean±SE) values of different experimental groups

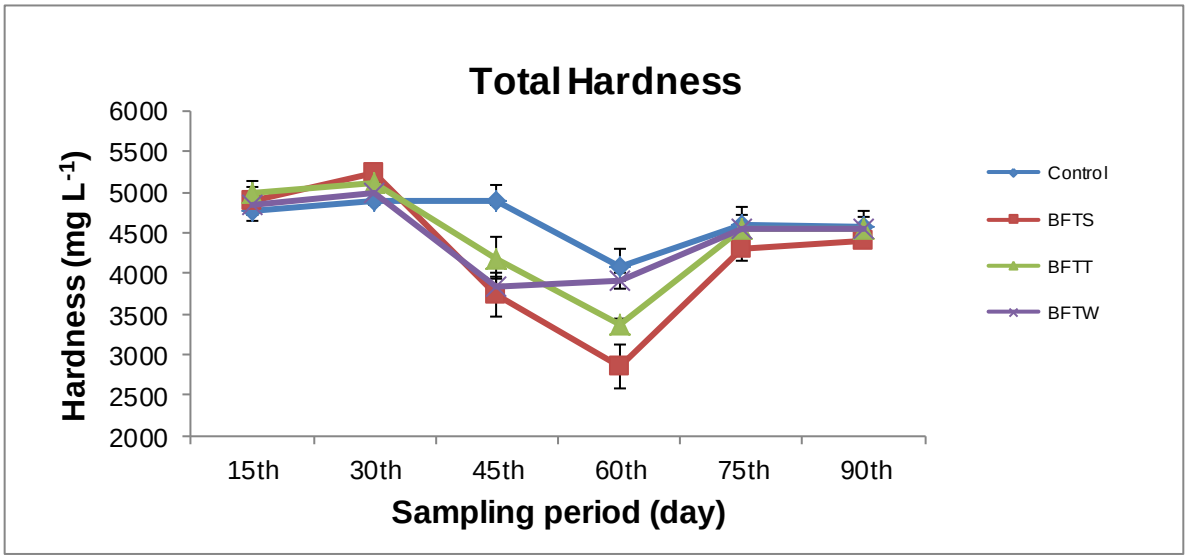

Fig. 11. Total hardness (mean±SE) in different experimental groups

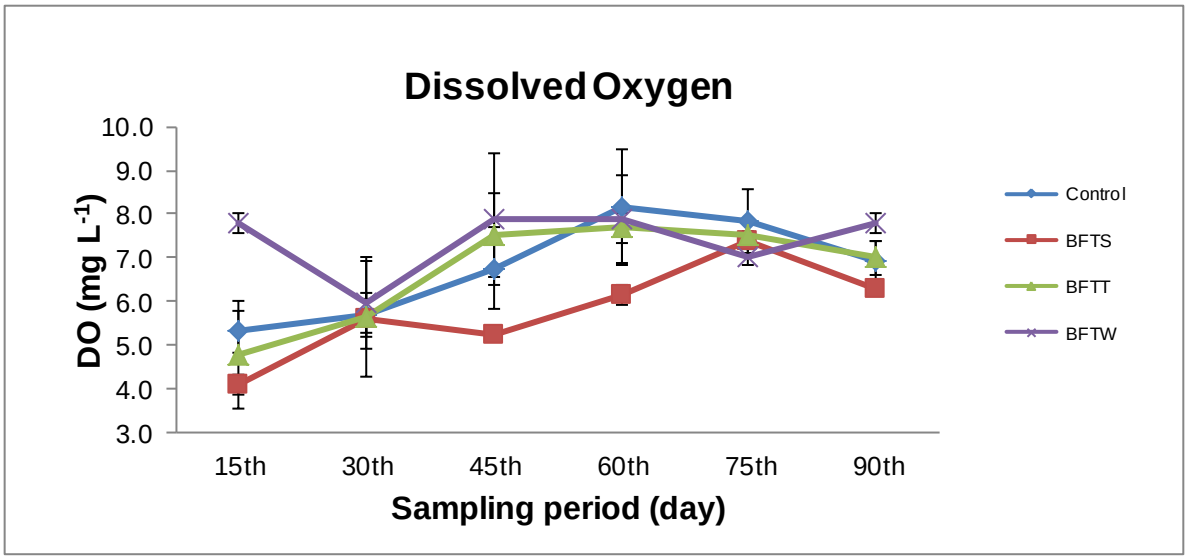

Fig. 12. Dissolved oxygen (mean±SE) fluctuation in different experimental groups

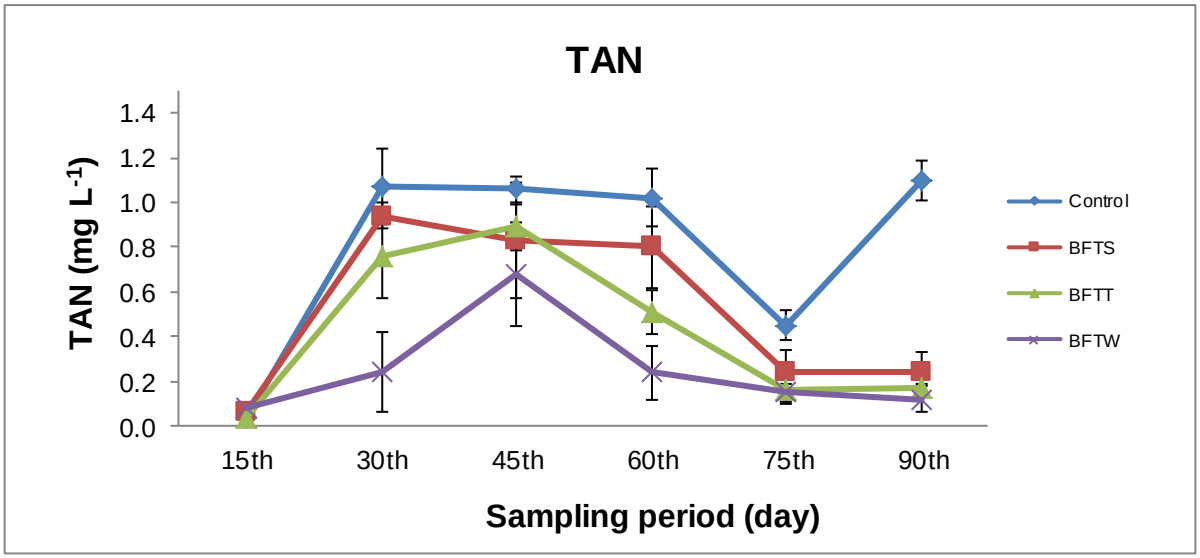

Fig. 13. TAN (mean±SE) fluctuation in different experimental groups

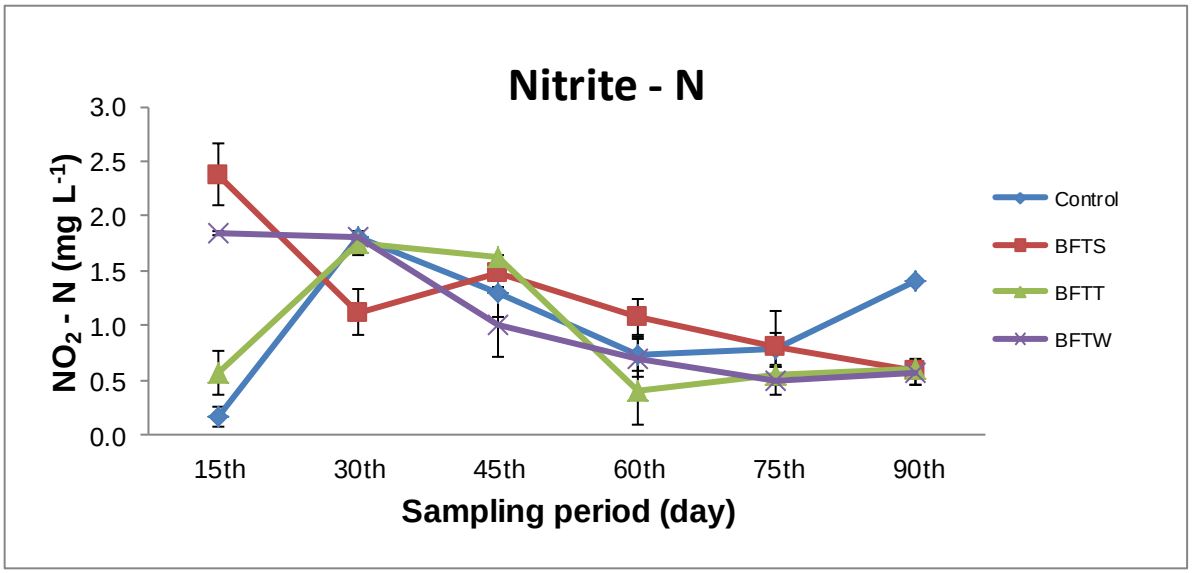

Fig. 14. Nitrite-N (mean±SE) fluctuation in different experimental groups

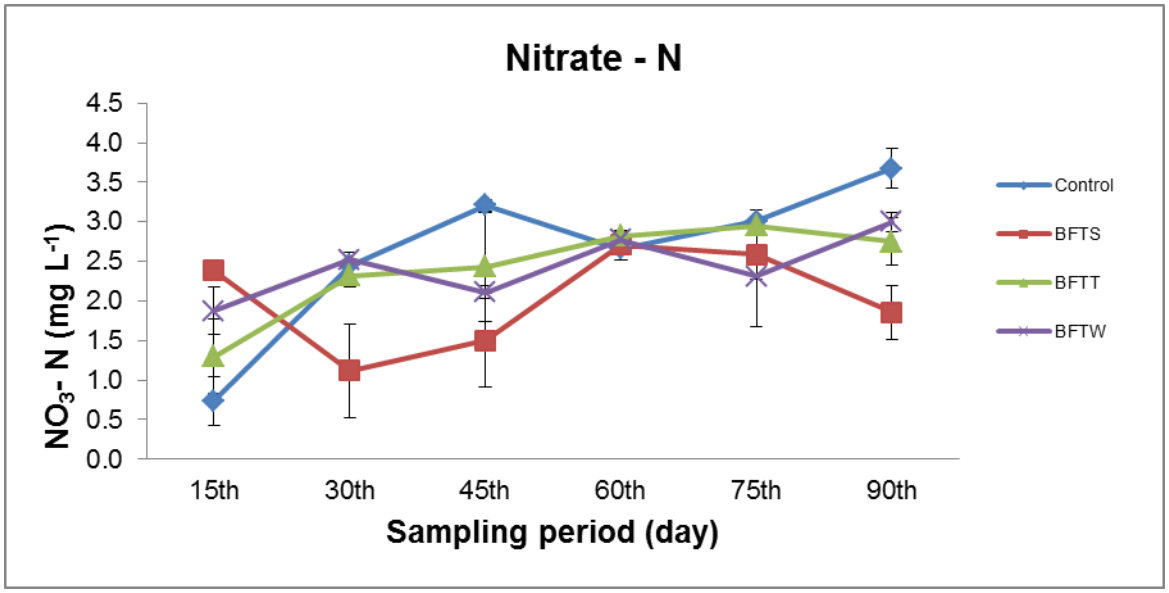

Fig. 15. Nitrate-N (mean±SE) fluctuation in different experimental groups

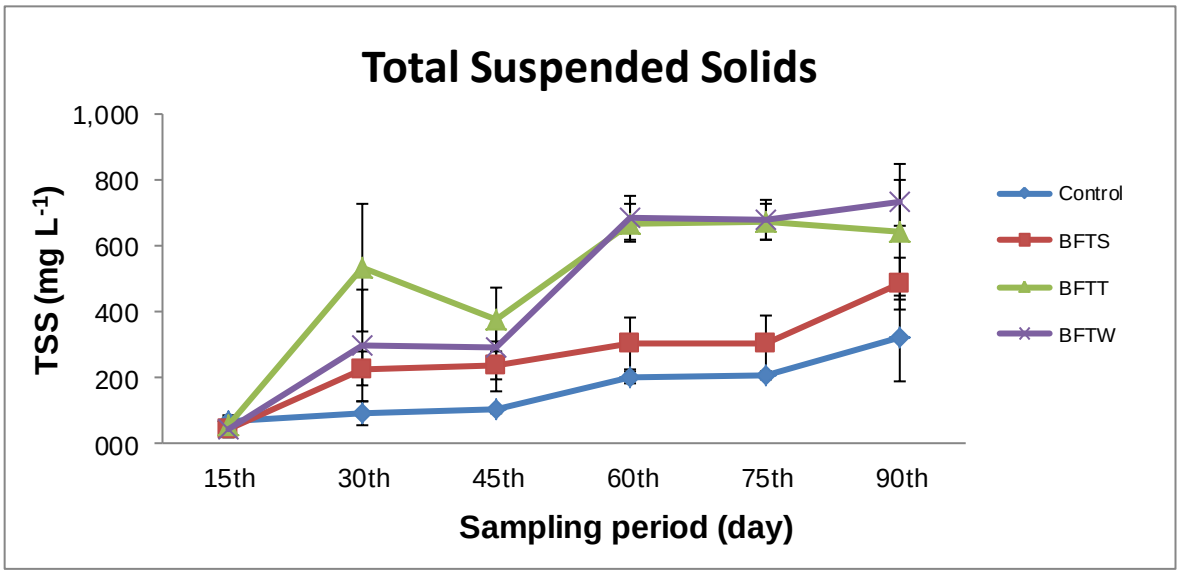

Fig. 16. Total suspended solids (mean±SE) fluctuation in different experimental groups

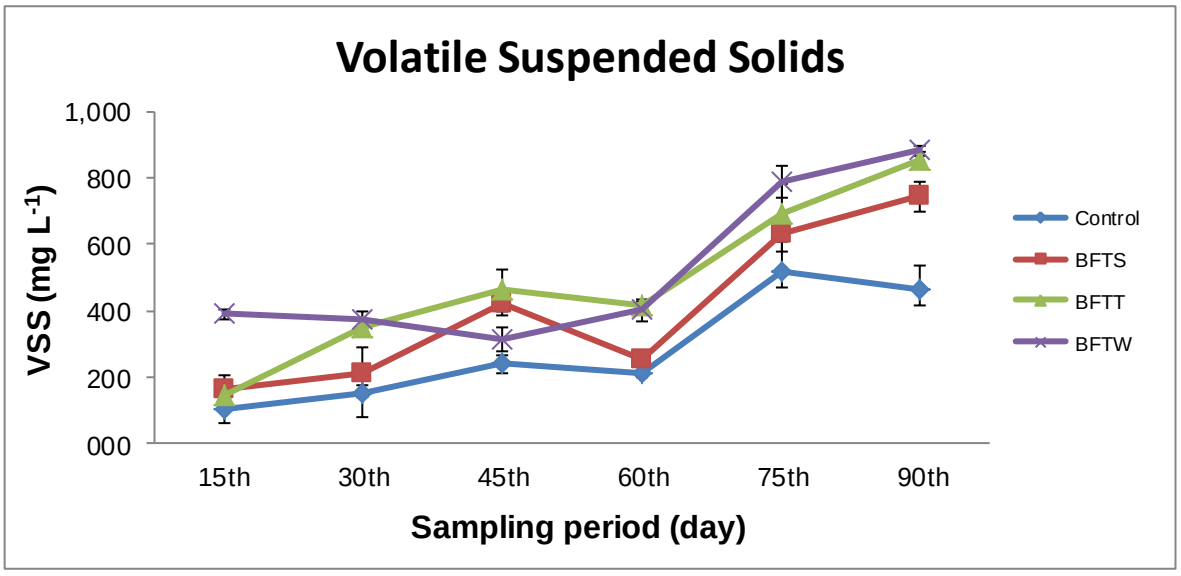

Fig. 17. Volatile suspended solids (mean±SE) fluctuation in different experimental groups

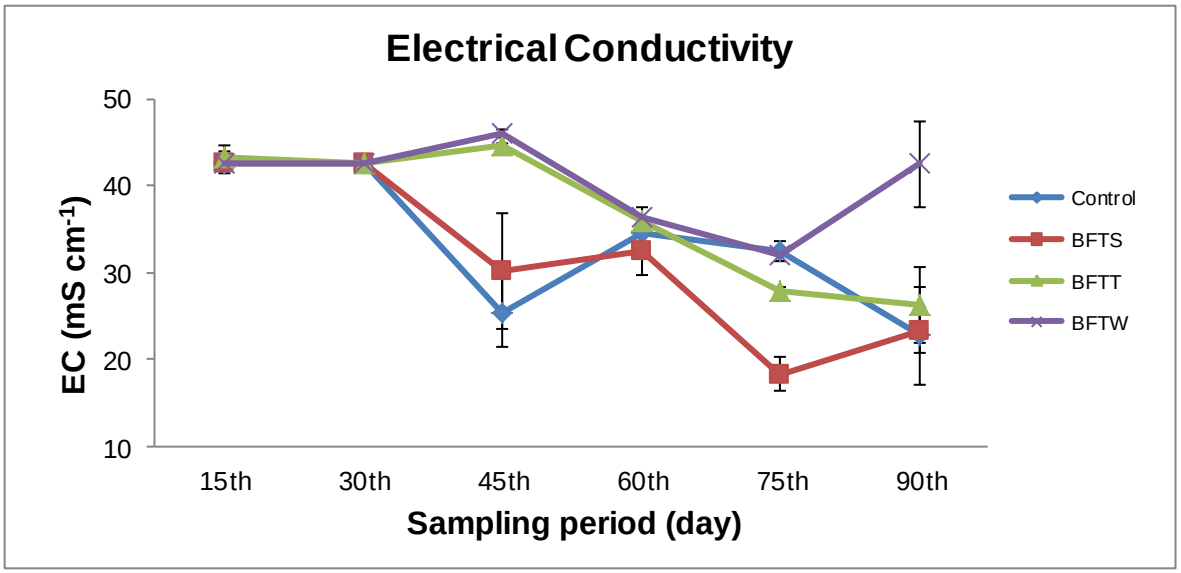

Fig. 18. EC (mean±SE) fluctuation in different experimental groups

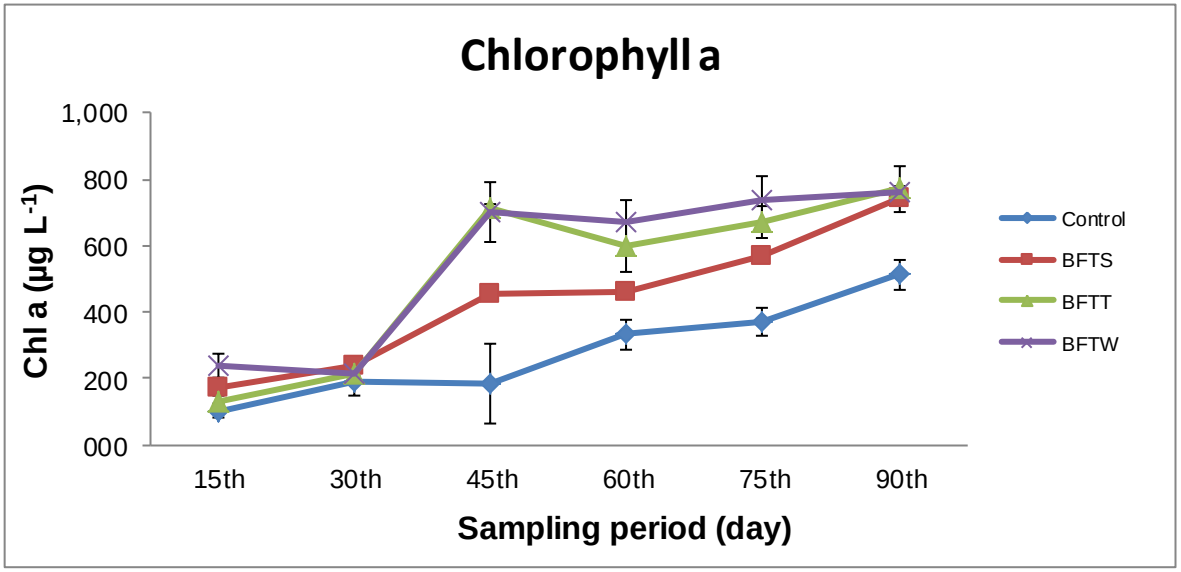

Fig. 19. Chlorophyll a (mean±SE) fluctuation in different experimental groups throughout the experimental period

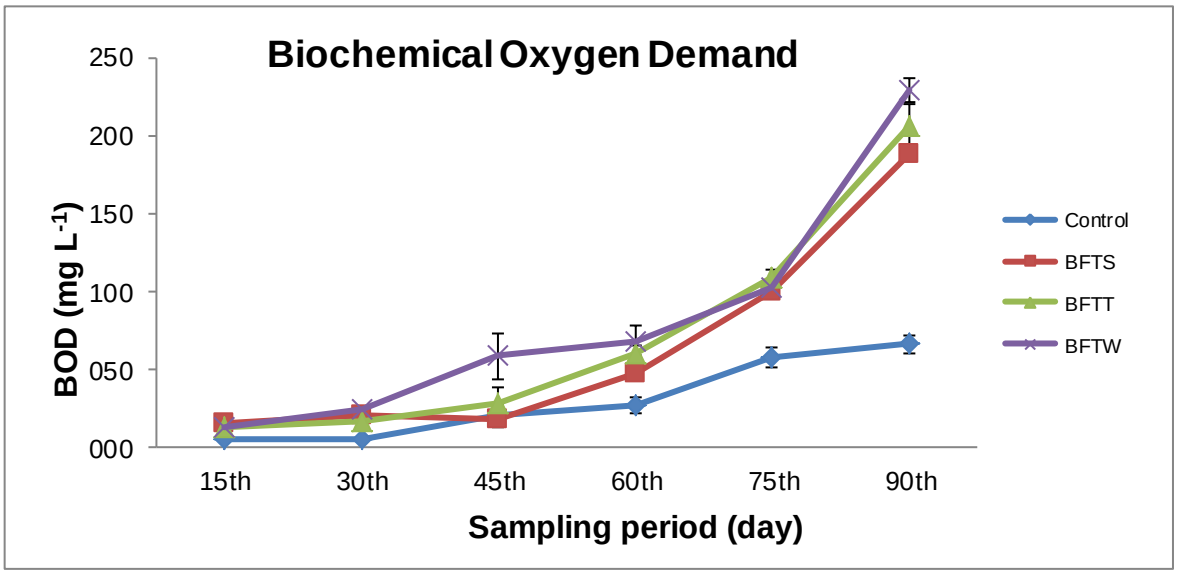

Fig. 20. BOD (mean±SE) fluctuation in different experimental groups

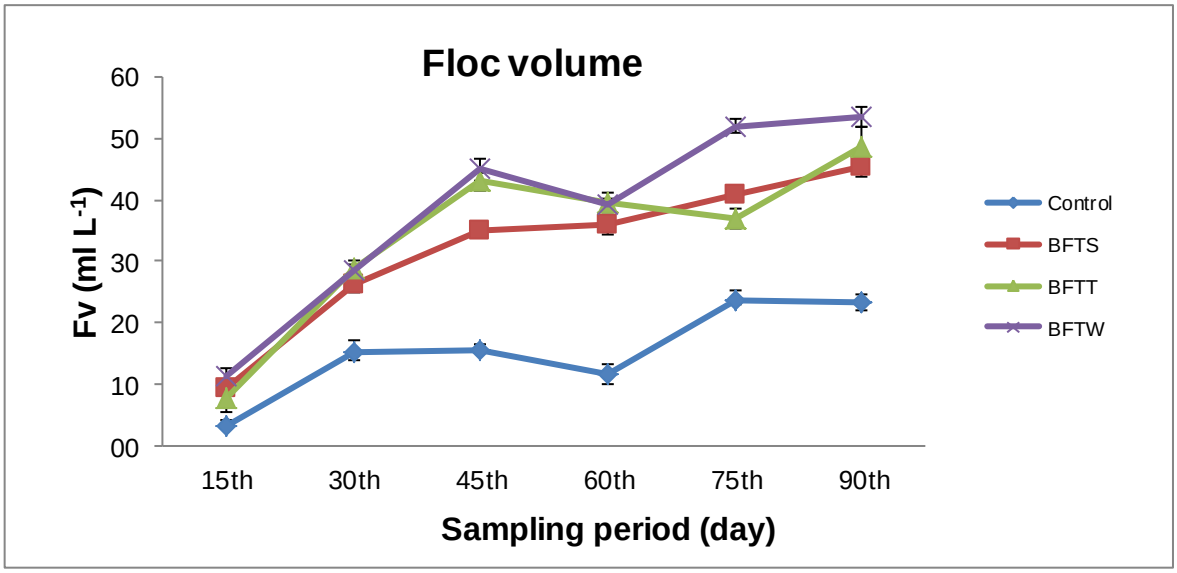

Fig. 21. Floc volume (mean±SE) fluctuation in different experimental group

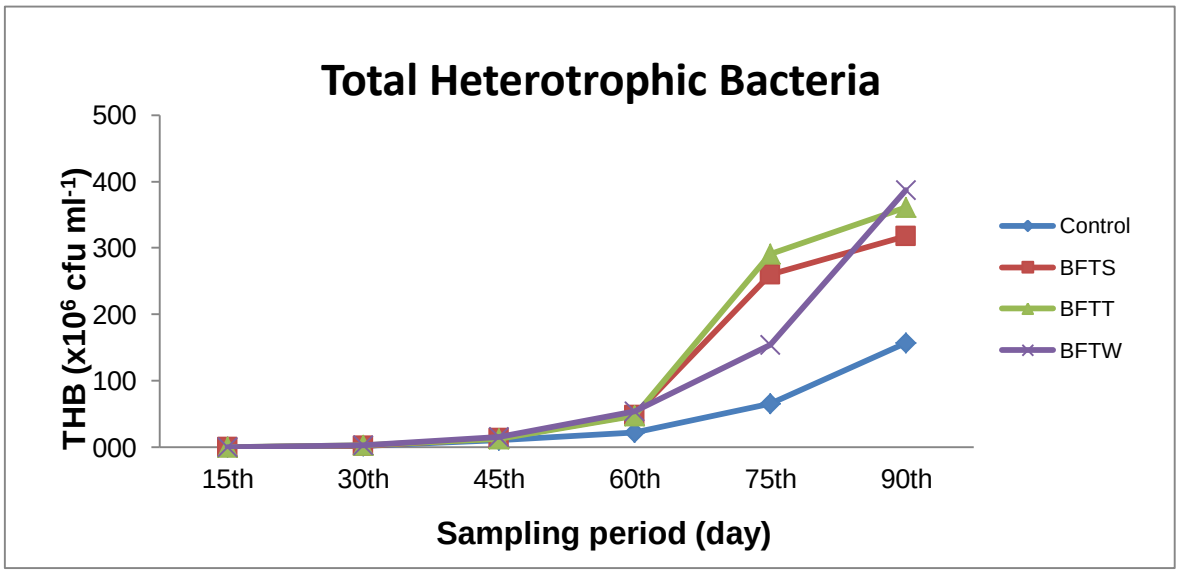

Fig. 22. THB (mean±SE) fluctuation in different experimental groups

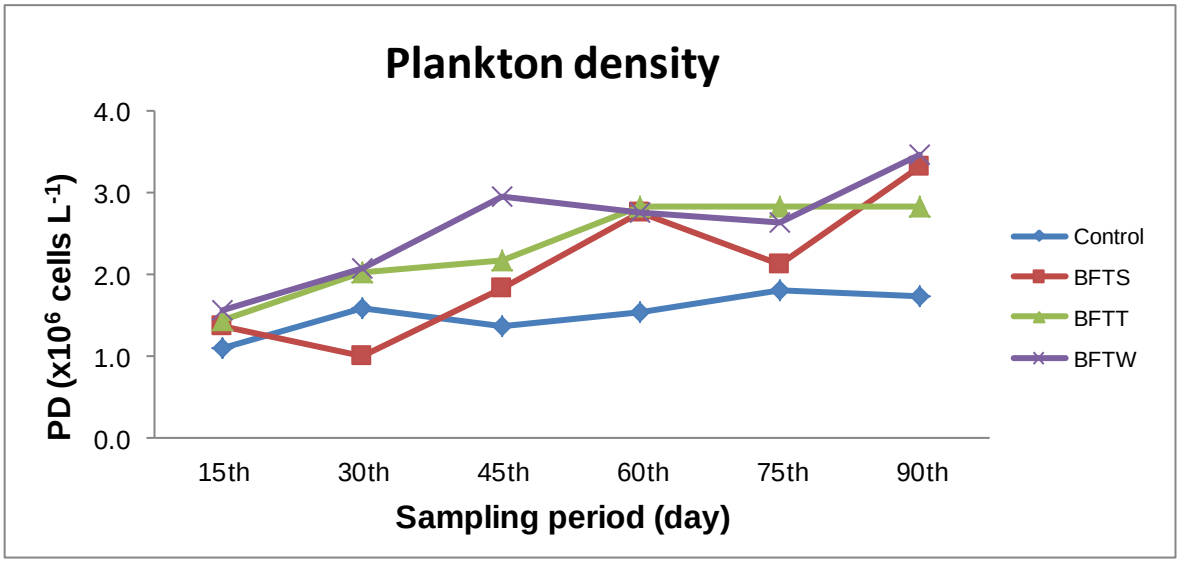

Fig. 23. Plankton density (mean±SE) fluctuation in different experimental groups

4.2.11. Electrical conductivity

The conductivity of the biofloc treatments ranged between 18.31 and 45.07 mS cm^{-1} (Fig. 18). EC did not vary significantly among treatments (Table 6).

4.2.12. Chlorophyll a

Chlorophyll-a values showed fluctuating trend between the treatments ranging from 102.93 to 772.41 µg L^{-1} (Fig. 19). All the biofloc treatments showed significantly higher chlorophyll a than the control (Table 6).

4.2.13. Biochemical oxygen demand

BOD increased after 75^{th} day in all the treatments except control (Fig. 20). BOD in BFT_W (108.4 mg L^{-1}) was significantly higher than that in BFT_S and control (Table 6)

4.2.14. Floc volume

Floc volume of BFT_W (43.6 ml L^{-1}) was significantly higher than other treatments. The BFT_T and BFT_S were at par in floc volume and had significantly higher than the control (Table 6, Fig. 21)

4.2.15. Total heterotrophic bacteria (THB)

The THB population during the experimental period in all the experimental groups, ranged from 6.0 x 10^2 to 3.87 x 10^8 cfu ml^{-1} (Fig. 22). The treatment BFT_W and BFT_T showed significantly higher mean THB concentration than the control (Table 6).

4.2.16. Plankton density

Plankton density showed fluctuating trend between treatments ranging from 1.0 to 3.46 x 10^6 cells L^{-1} (Fig. 23). Significantly higher plankton density was recorded in BFT_W (2.38 x 10^6 cells L^{-1}) and BFT_T (2.23 x 10^6 cells L^{-1}) than that in control (1.45 x 10^6 cells L^{-1}). BFT_S did not differ than control as well as than other biofloc treatments (Table 6).

4.3. Morphology and Plankton composition of biofloc

The structure of biofloc was observed under a microscope with 10X objective. In the bioflocs treatment group, floc was seen in anomalous flocculation with bacteria and zooplankton especially nematodes (Fig. 24). However, in the relative control group, there were a few of detritus and sloughs in the sediment of the Imhoff cone. The taxonomic compositions of different floc-associated planktonic organisms are given in Fig. 25. The group of organisms were identified: tintinids, ciliates, copepods cyanobacteria, and nematodes. Among the groups of organism, nematodes were the most dominant in all the biofloc treatment tanks. The total numbers of organism were significantly higher in the BFT_W followed by BFT_T and control. These microorganisms were found to be grazing on the flocs, when fresh samples were observed under microscope under microscope. In all the biofloc treatment tanks, mostly the zooplanktons were dominant and very limited number of phytoplankton could be visualized under the microscope. The only one phytoplankton (*Spirulina* sp.) was observed along with zooplankton.

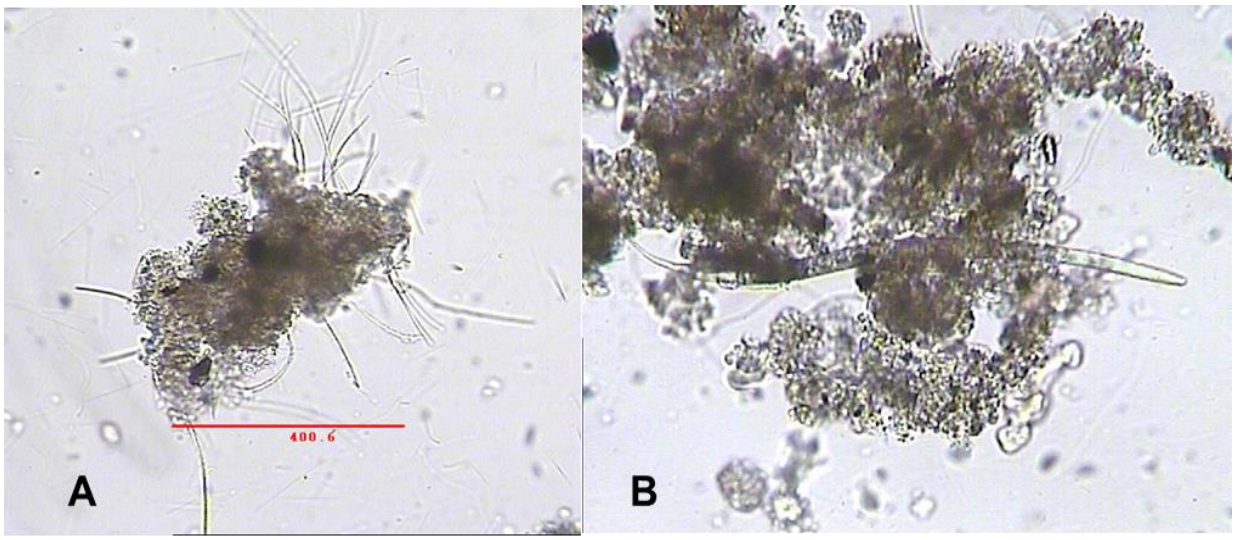

Fig. 24. Morphology of floc under microscope (A) biofloc particle flocculation with filamentous algae and bacteria (B) biofloc flocculates with nematodes

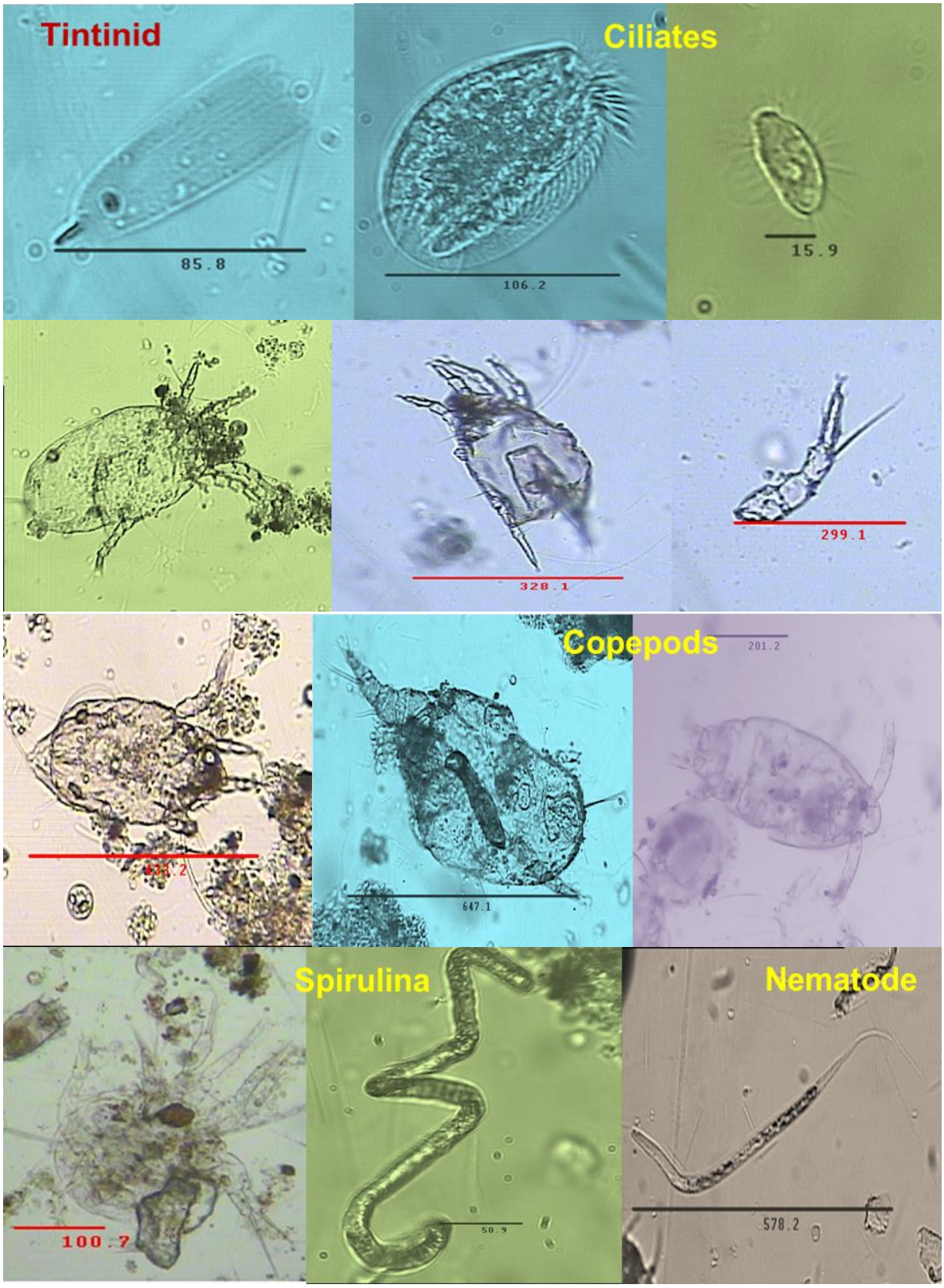

Fig. 25. Common zooplankton and phytoplankton observed in the biofloc treatments

4.4. Growth Parameters

4.4.1. Average Body Weight

The body weight of the experimental groups was recorded at fortnightly interval is shown in Fig. 31. There was a significant difference ($P<0.05$) in the average body weight among treatments and control (Table 8). The highest growth was observed in the group BFT_W and the lowest was in the control group. The initial body weight among the treatment group varied from 0.137±0.001 to 0.150±0.005 g and the final body weight varied from 5.97±0.03 to 8.49±0.09 g (Table 7, Fig. 26). The treatment groups has more weight gain BFTs (29.7%), BFT_T (17.7%), BFT_W (10.1%) compared to control.

4.4.2. Growth rate

The growth rate was showed increasing trend during the experimental period in the biofloc treatments and control (Fig. 27). The growth rate significantly ($P<0.05$) varied among biofloc treatments as well as between different treatments and control (Table 8). The significantly lowest (0.06±0.00 g day^{-1}) growth rate was recorded in control and the significantly higher growth rate was found in BFT_W (0.09±0.001 g day^{-1}).

4.4.3. Feed Conversion Ratio (FCR)

The feed conversion ratio value significantly ($P<0.05$) varied among biofloc treatments and the control (Fig. 28). The significantly higher and best FCR was recorded in the treatment BFT_W (1.03±0.03) and the significantly lower FCR was found in control (1.69±0.01) followed by BFT_S (Table8).

Table 7: Average body weight (mean±SE, n = 3) of the different experimental groups during the experimental period

Treatment	0^{th} day	15^{th} day	30^{th} day	45^{th} day	60^{th} day	75^{th} day	90^{th} day
Control	0.150±0.005	0.474^{a}±0.050	1.188^{a}±0.120	2.042^{a}±0.130	2.955^{c}±0.160	4.344^{a}±0.350	5.973^{d}±0.030
BFT_S	0.148±0.002	0.451^{ab}±0.020	1.050^{ab}±0.220	2.176^{bc}±0.320	3.142^{c}±0.140	4.781^{a}±0.310	6.644^{c}±0.130
BFT_T	0.137±0.001	0.532^{ab}±0.020	0.969^{ab}±0.080	2.537^{ab}±0.090	3.206^{b}±0.090	4.935^{a}±0.240	7.25^{b}±0.090
BFT_W	0.141±0.003	0.592^{b}±0.040	1.440^{b}±0.060	3.222^{a}±0.120	4.176^{a}±0.130	5.223^{a}±0.200	8.486^{a}±0.090

The mean value (g) in a column bearing different superscript differs significantly ($p < 0.05$).

Table 8. Growth parameters of shrimp in different biofloc treatments and the control (means±SE, n=3) at the end of the experiment

Parameters	Control	BFT_S	BFT_T	BFT_W	P value
ABW	5.97^{d}±0.03	6.64^{c}±0.13	7.25^{b}±0.09	8.49^{a}±0.09	<0.0001
Growth rate (g day^{-1})	0.06^{d}±0.00	0.07^{c}±0.001	0.08^{b}±0.002	0.09^{a}±0.001	<0.0001
SGR	4.10^{d}±0.03	4.25^{c}±0.04	4.43^{b}±0.02	4.57^{a}±0.03	<0.0001
FCR	1.69^{a}±0.01	1.24^{b}±0.02	1.14^{c}±0.01	1.03^{d}±0.03	<0.0001
PER	1.58^{d}±0.01	2.16^{c}±0.03	2.34^{b}±0.03	2.59^{a}±0.08	<0.0001
FER	4.1^{d}±0.03	4.25^{c}±0.04	4.43^{b}±0.02	4.57^{a}±0.03	<0.0001
Survival (%)	86.9^{a}±3.8	82.2^{a}±3.4	85.6^{a}±3.5	90.3^{a}±0.96	0.205
Total weight (g)	639.03^{c}±13.27	670.67^{c}±27.31	758.69^{b}±38.17	934.65^{a}±15.03	0.0002
Total weight gain (g)	623.00^{c}±12.99	656.08^{c}±27.14	744.63^{b}±37.64	919.41^{a}±14.76	0.0001

ABW: Average body weight gain; **SGR:** Specific Growth Rate; **FCR:** Feed Conversion Ratio; **FER:** Feed efficiency ratio; **PER:** Protein Efficiency Ratio. Mean values in the same row with different superscript differ significantly ($P<0.05$).

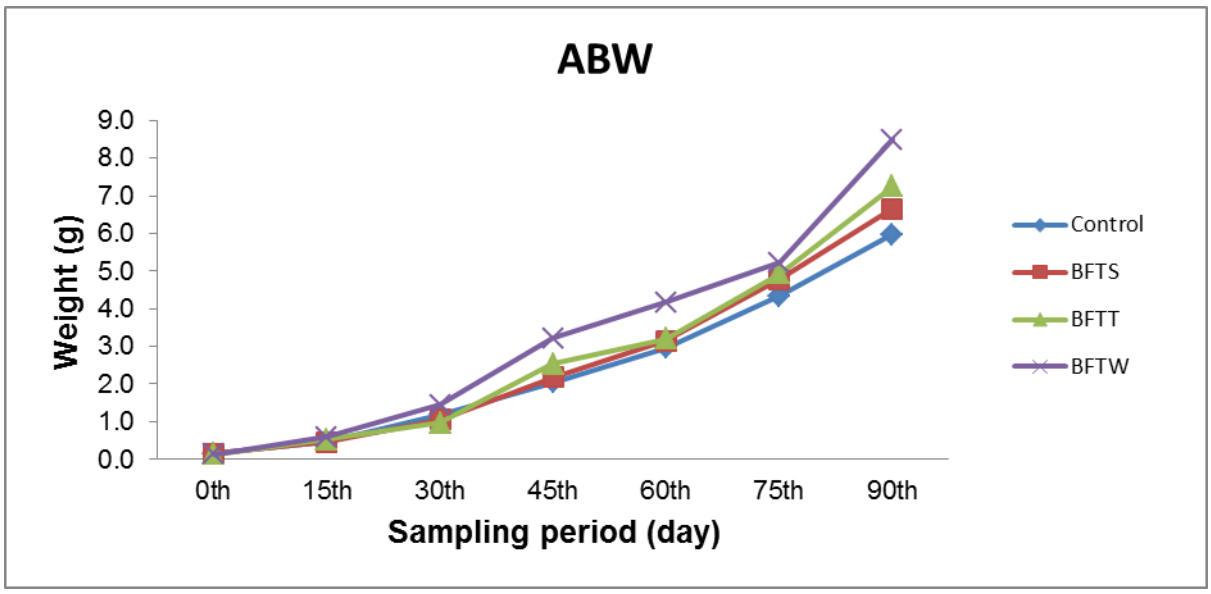

Fig. 26. Average body weight (mean±SE) in the different treatments and the control

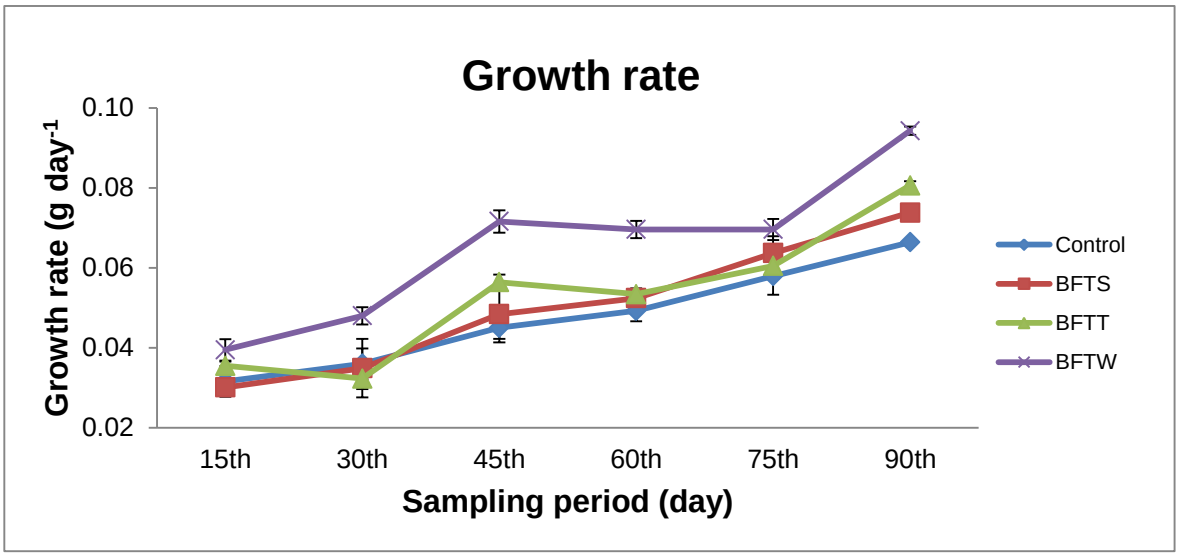

Fig. 27. Growth rate (mean±SE) in the different treatments and the control

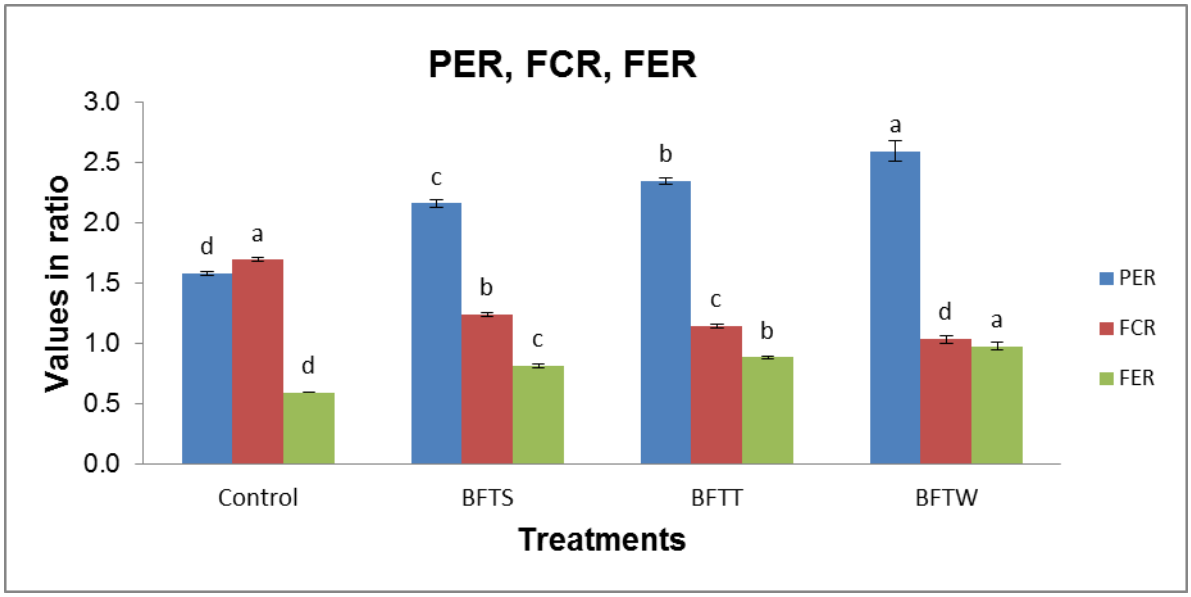

Fig. 28. PER, FCR and FER values (mean±SE) in the different treatments and the control. Different letters on the bar among the same parameter vary significantly (P<0.05).

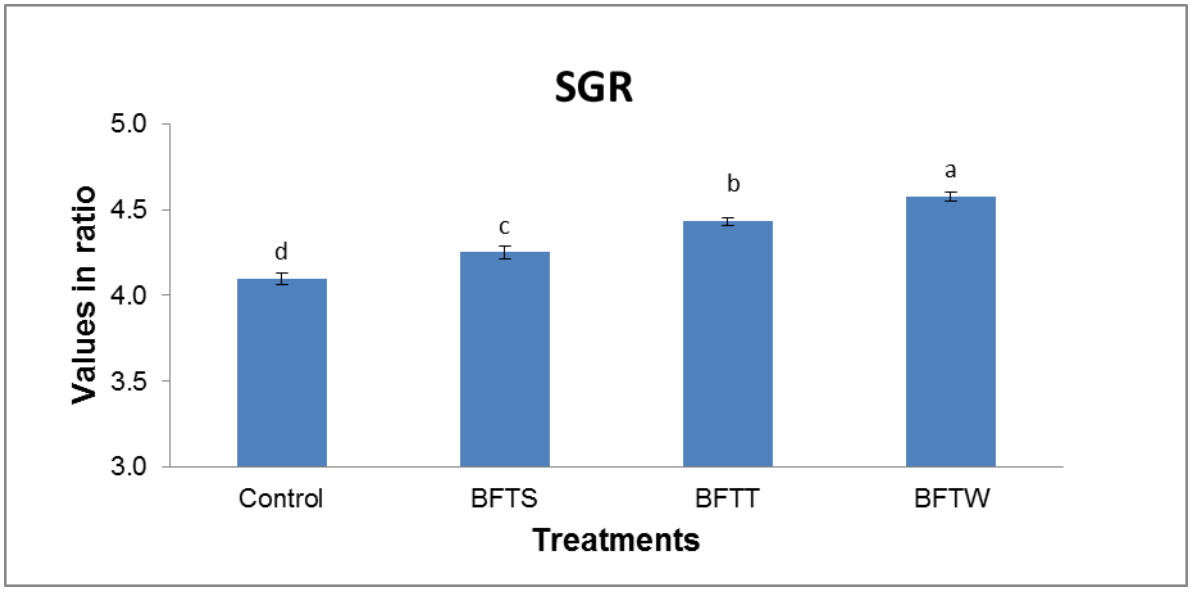

Fig. 29. SGR values (mean±SE) in the different treatments and the control. Different letters on the bar vary significantly (P<0.05).

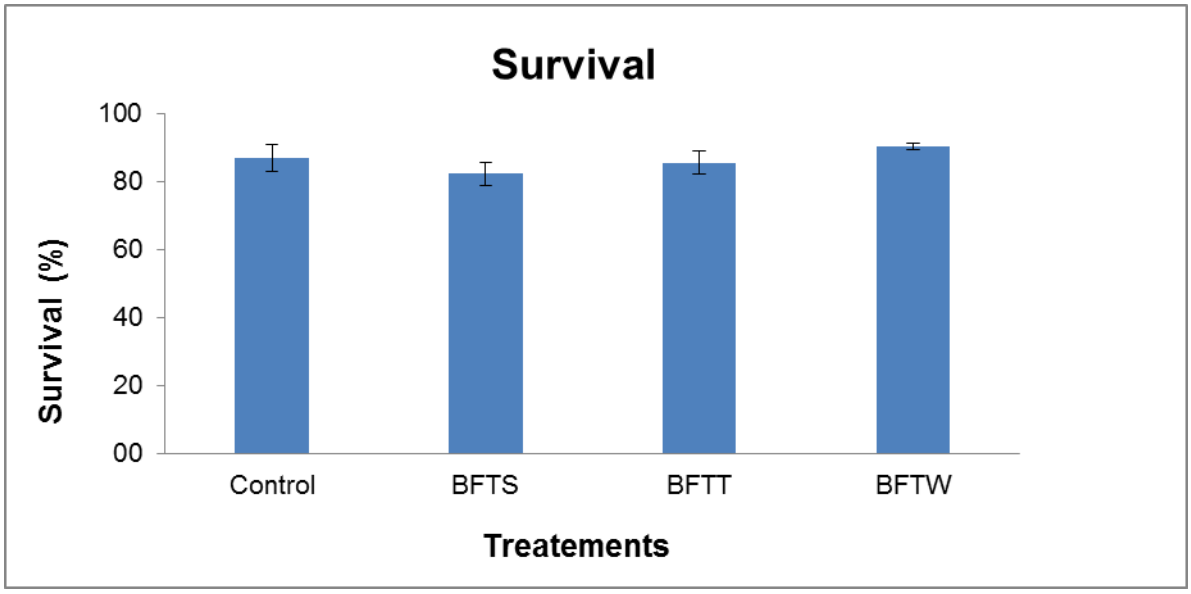

Fig. 30. Survival (%) in different experimental groups. Survival did not vary significantly among experimental groups (P>0.05).

4.4.4. Protein Efficiency Ratio (PER)

The mean PER value was significantly different (P<0.05) among the different biofloc treatment groups as well as between the treatments and the control (Fig. 28). The significantly higher PER value was recorded in BFT_W (2.59±0.08) and the significantly lowest PER was recorded in control (1.58±0.01) (Table 8).

4.4.5. Specific Growth Rate (SGR)

The value of SGR varied significantly (P<0.05) among different treatment groups (Fig. 29). The significantly lowest SGR value was found in the control (4.10±0.03) group and significantly higher SGR was found in the treatment BFT_W (4.57±0.03) (Table 8).

4.4.6. Feed Efficiency Ratio (FER)

The mean FER value was significantly different (P<0.05) among the treatments and control (Fig. 28). The significantly highest FER value was recorded in BFT_W (4.57±0.03) and the significantly lowest FER was recorded in control (4.1±0.03) (Table 8).

4.4.7. Survival Rate

The survival rates of the animals in the different experimental groups are given in the table 8 and figure 30. There was no significant difference ($p>0.05$) in survival rate between the biofloc treatments and control. The mean survival rate was found to be 86%.

4.5. Proximate Composition of Shrimp

The crude protein, ether extract, crude fiber, NFE, TC and ash content of the shrimp was significantly ($P<0.05$) differed among the treatments and control but the moisture content of experimental animals did not vary significantly (table 9). The significantly higher crude protein was found in BFT_W (51.09±2.0%) and significantly lower crude protein was found in control (39.44±0.18). The significantly higher EE was found in BFT_W (10.61±0.29%) and significantly lower EE was found in BFT_S (7.24±0.41%). The significantly higher CF was found in control (8.26±0.31%) and significantly lower CF was found in BFT_S (6.95±0.30%). The significantly higher NFE was found in BFT_S (37.35±1.08%) and significantly lower NFE was found in BFT_W (22.93±2.12%). The significantly higher TC was found in control (44.55±0.3%) and significantly lower TC was found in BFT_W (31.15±2.16%). The significantly higher ash was found in control (8.34±0.15%) and significantly lower ash was found in BFT_T (6.68±0.07%).

4.6. Nutritional quality of biofloc

4.6.1. Proximate composition of biofloc

The crude protein, ether extract, crude fiber, NFE, TC, ash, C: N ratio and gross energy of the bioflocs were significantly ($P<0.05$) differed among the treatments and control but the moisture content of the biofloc did not varied significantly ($P>0.05$) (table 10). The significantly higher crude protein was found in BFT_W (53.65±0.7%) and significantly lower crude protein was found in control (25.29±0.75). The significantly higher EE was found in BFT_W (0.92±0.003%) and significantly lower EE was found in BFT_T (0.57±0.07%) and control (0.48±0.09%). The significantly higher CF was found in BFT_W (16.65±0.02%) and significantly lower CF was found in control (11.88±1.10%). The significantly higher NFE was

found in control (33.32±2.87%) and significantly lower NFE was found in BFT_W (3.74±0.43%). NFE was significantly lower in the treatments than control. The significantly higher TC was found in control (45.20±1.77%) and significantly lower TC was found in BFT_W (20.39±0.45%). TC did not significantly vary between BFT_T (32.39±1.93%) and BFT_S (30.92±0.45). The significantly higher ash was found in BFT_T (14.88±0.46%) and significantly lower ash was found in BFT_S (22.53±0.22%). The significantly higher C:N ratio was found in BFT_W and BFT_T (5.41±0.1 and 5.25±0.16) and significantly lower C:N was found in control (2.55±0.08). The significantly higher GE was found in BFT_W (27.25±0.86 kJ g^{-1}) and significantly lower GE was found in control (19.42±1.02 kJ g^{-1}).

4.6.2. Mineral composition of biofloc

The mineral composition of biofloc derived from the tanks fed with different carbon sources is given in Table 11. There were significant differences (<0.05) in mineral composition such as Fe, Zn, Ca, Mg, Na, K, and total phosphorus between the biofloc treatments and the control. The higher mineral content was found in the biofloc treatments than control.

4.7. Interaction between biofloc proximate composition and shrimp growth parameters

The correlation matrix between biofloc proximate composition and shrimp growth parameters between the treatments showed many significant correlations (Table 12). The significant positive correlation was obtained between crude protein growth rate, average body weight, total weight, specific growth rate and feed efficiency ratio, whereas some significant negative correlations were observed between the crude protein and feed conversion ratio.

Table 9. Proximate composition of *L. vannamei* cultured in different biofloc treatments and the control (% means±SE, n=3) at the end of the experiment

Parameters	Control	BFT_S	BFT_T	BFT_W	P value
Moisture (%)	76.82^{a}±0.78	76.20^{a}±0.14	75.91^{a}±2.38	78.53^{a}±2.0	0.3012
Ash (%)	8.34^{a}±0.15	7.15^{b}±0.56	6.68^{b}±0.07	7.15^{b}±0.11	0.0229
Crude Protein (%)	39.44^{c}±0.18	41.31^{c}±0.73	47.50^{b}±0.06	51.09^{a}±2.0	0.0002
Ether Extract (%)	7.67^{c}±0.18	7.24^{c}±0.41	9.04^{b}±0.58	10.61^{a}±0.29	0.0012
Crude Fiber (%)	8.26^{a}±0.31	6.95^{b}±0.3	7.8^{a}±0.12	8.22^{a}±0.14	0.0128
NFE (%)	36.29^{a}±0.39	37.35^{a}±1.08	28.98^{b}±0.7	22.93^{c}±2.12	0.0001
TC (%)	44.55^{a}±0.3	44.3^{a}±1.25	36.78^{b}±0.6	31.15^{c}±2.16	0.0002

NFE: Nitrogen Free Extract; **TC:** Total Carbohydrate. Values other than moisture are expressed on dry weight basis. Mean values in the same row with different superscript differ significantly ($P<0.05$).

Table 10. Nutritional composition of biofloc in different biofloc treatments and the control (% means±SE, n=3) at the end of the experiment

Parameters	Control	BFT_S	BFT_T	BFT_W	P value
Moisture (%)	74.34^{a}±5.43	63.51^{ab}±2.14	63.74^{ab}±2.51	56.51^{b}±3.12	0.096
Ash (%)	29.03^{a}±0.93	22.53^{c}±0.22	14.88^{d}±0.46	25.04^{b}±0.39	0.000
Crude Protein (%)	25.29^{c}±0.75	45.98^{b}±0.59	52.03^{a}±1.57	53.65^{a}±0.7	0.000
Ether Extract (%)	0.48^{b}±0.09	0.57^{b}±0.07	0.70^{ab}±0.03	0.92^{a}±0.003	0.027
Crude Fiber (%)	11.88^{c}±1.10	12.92^{bc}±0.8	15.25^{ab}±0.06	16.65^{a}±0.02	0.024
NFE (%)	33.32^{a}±2.87	18.00^{b}±0.35	17.14^{b}±1.87	3.74^{c}±0.43	0.001
TC (%)	45.20^{a}±1.77	30.92^{b}±0.45	32.39^{b}±1.93	20.39^{c}±0.45	0.001
CN ratio	2.55^{c}±0.08	4.64^{b}±0.06	5.25^{a}±0.16	5.41^{a}±0.1	0.000
GE (kJ g^{-1})	19.42^{c}±1.02	22.45^{c}±0.56	25.4^{b}±1.25	27.25^{a}±0.86	0.0002

GE: Gross energy. Values other than moisture are expressed in dry weight basis. Mean values in the same row with different superscript differ significantly ($P<0.05$)

Table 11. Mineral composition (dry weight basis) of biofloc in different biofloc treatments and the control (means±SE, n=3) at the end of the experiment

Parameters	Control	BFT_S	BFT_T	BFT_W	P value
Fe (mg kg^{-1})	4918.33^{b}±69.17	4417.33^{c}±45.04	11200^{a}±179.50	4479^{bc}±204.86	<0.0001
Zn (mg kg^{-1})	161.5^{b}±0.43	146.2^{c}±0.40	200.77^{a}±0.26	200.63^{a}±0.58	<0.0001
Ca (mg g^{-1})	18.9^{c}±1.04	23.9^{b}±3.78	37.1^{a}±3.66	22.7^{b}±2.070	<0.0001
Mg (mg kg^{-1})	3537^{d}±7.77	7315^{b}±61.582	6302.33^{c}±10.14	12300^{a}±124.23	<0.0001
Na (mg g^{-1})	15.45^{b}±2.73	16.52^{a}±1.91	15.71^{b}±1.81	16.81^{a}±1.82	0.0029
K (mg kg^{-1})	5773.87^{c}±60.84	6162.7^{b}±75.51	6057.21^{b}±66.93	7982.96^{a}±58.83	<0.0001
TP (mg g^{-1})	14^{b}±2.00	23.8^{ab}±1.20	27^{a}±3.0	31.5^{a}±3.50	<0.0001

Fe: Iron; Zn: Zinc; **Ca:** Calcium; Mg**:** Megnesium; **Na:** Sodium; **K:** Potassium; **TP:** Total Phosphorus. Mean values in the same row with different superscript differ significantly (P<0.05)

Table 12. Correlation matrix of biofloc proximate composition with growth parameters

Parameters	M	ASH	CP	EE	CF	NFE	TC	ABW	GR	SGR	FCR	PER	FER	S	TW
M															
Ash	-0.22														
CP	0.27	-0.55*													
EE	0.46	-0.21	0.88*												
CF	0.32	0.4	0.19	0.45											
NFE	-0.33	0.33	-0.96*	-0.96*	-0.41*										
TC	-0.31	0.39	-0.98*	-0.95*	-0.32	0.99*									
ABW	0.44	-0.51	0.88*	0.83*	0.25	-0.87*	-0.87*								
GR	0.44	-0.51	0.88*	0.83*	0.25	-0.87*	-0.87*	1							
SGR	0.43	-0.62*	0.91*	0.81*	0.17	-0.86*	-0.88*	0.96*	0.96*						
FCR	-0.3	0.75*	-0.82*	-0.61*	0.16	0.7*	0.74*	-0.86*	-0.86*	-0.91*					
PER	0.29	-0.7*	0.88*	0.7*	-0.06	-0.7*8	-0.82*	0.91*	0.91*	0.94*	-0.99*				
FER	0.29	-0.7*	0.88*	0.7*	-0.06	-0.78*	-0.82*	0.91*	0.91*	0.94*	-0.99*	1			
Survival	0.15	-0.1	0.35	0.26	0.39	-0.37	-0.34	0.43*	0.43	0.38	-0.11	0.19	0.19*		
TW	0.4	-0.44	0.83*	0.76*	0.33	-0.83*	-0.83*	0.96*	0.96*	0.91*	-0.74*	0.8*	0.8	0.66*	
TWG	0.4	-0.44	0.84*	0.77*	0.32	-0.83*	-0.82*	0.96*	0.96*	0.91*	-0.74*	0.81*	0.81	0.66*	0.99*

(*****: significant correlations; **M:** Moisture; **CP:** Crude Protein; **EE:** Ether Extract; **CF:** Crude Fiber; **NFE:** Nitrogen free extract; **TC:** Total carbohydrate; **ABW:** Average body weight gain; **GR:** Growth rate; **SGR:** Specific growth rate; **FCR:** Feed conversion ratio; **PER:** Protein efficiency ratio; **FER:** Feed efficiency ratio; **TW:** Total weight; **TWG:** Total weight gain

5. DISCUSSION

5.1. Microbial community in biofloc

Analysis of DGGE bands from biofloc of the different treatments revealed the existence of a fairly diverse bacterial community. The total number of bands visualized in a DGGE gel also provides an estimate of the bacterial community found within a biofloc treatment (Zhao *et al.,* 2012). During the study, 13 different sequences were obtained from BFT_S sediment, 12 from BFT_T, 15 from BFT_W and 5 from control. The comparison of the 16S rRNA gene sequence derived from the DGGE bands with those of GenBank database revealed the identity of the bacteria. From sequence analyses, *Ruegeria* sp. *Chitinophaga pinensis, Roseobacter litoralis, Hyphomonas neptunium* and *Phaeobacter gallaeciensis* were found to be the predominant bacteria in all the treatments.

Isolation and culture of majority of the associated microflora in shrimp and other organism has proved difficult and therefore, research has focused on growth independent identification techniques (Lau *et al*., 2002) or those techniques designed to monitor and identify particular microorganisms (Tendencia and de la Pena, 2001; Nakayama *et al*., 2006). DGGE is one of the growth independent techniques which are used for assessing the microbial diversity in biofloc (Zhao *et al*., 2012). Profiling of microbial communities in soil, marine environments, hydrothermal vents, gastrointestinal tract of humans, bioflocculation in wastewater and even the study of a microbial community resident in a medieval wall painting (Felske *et al.,* 1997; Hold *et al.,* 2001; Zoetendal *et al.,* 2002; Nicol *et al.,* 2003; Xia *et al*., 2005) have already been done using the DGGE technique. The recent studies have shown the dominance of gram positive bacteria than gram negative bacteria in the biofloc system (Haslun *et al*., 2012). The *Bacillus* sp. and *Proteobacterium* sp. were characterized in biofloc system and *Vibrio* sp. in control system (Zhao *et al.,* 2012). Many bacterial communities were found but there is no *Bacillus, Proteobacterium* and *Vibrio* sp. could be identified. The presence of bacterium *Roseobacter* sp. in all the treatments might be responsible for the elimination of

Vibrio in the system. Because *Roseobacter* sp. is lethal to the shrimp larval pathogens *Vibrio* sp. in a buffer system and they have tendency to inhibit their growth (Bruhn *et al*., 2005). The distribution of dominant microorganisms and resilient ones in the biofloc system assures the competence of counteracting the effect of a sudden exposure to disturbance. These preliminary molecular results indicate that the field of molecular analysis of the BFT needs further exploration

5.2. Water quality parameters

Water temperature (22.0-24.8°C) observed during the experimental period was in the optimal range. The salinity was maintained in about 25‰ throughout experimental period. Salinity is probably not the sole limiting factor for growth in cultured white shrimp. Bray *et al.* (1994) showed that 5 and 15‰ salinity produced significantly greater final weights than other levels tested and the hyper-saline treatment (49‰) showed significantly lesser growth of *L. vannamei* than other treatments. Ponce-Palafox *et al.* (1997) reported that within the optimum temperature range, the tolerance of *L. vannamei* to salinity is wide, and good growth can be obtained between 25 and 45‰. It's clear that Pacific white shrimp can survive up to 0‰ but the best growth was found in the salinity range of 15 to 30‰. Van Wyk *et al*. (1999) reported that salinities less than 0.5‰ showed adverse effect on growth, preventing it from reaching commercial size.

The lowest level of dissolved oxygen observed in this study (4.07 mg L^{-1}) was high enough for good survival and growth of *L. vannamei* (Aquacop *et al.,* 1988; McGraw *et al*., 2001). This was probably due to continuous aeration in biofloc tanks. Low DO in biofloc tanks in comparison to control indicated that microbes consumed oxygen for decomposing organic matter, as well as for respiration. The lower pH values in the biofloc tanks were possibly a result of high respiration rates by the large quantities of microorganisms (heterotrophic community), which might have increased CO_2 concentrations. A similar trend was observed by Tacon *et al*. (2002) and Wasielesky *et al*. (2006). In addition, Chen *et al*. (2006), Ebeling *et al.* (2006) and Rijn *et al.* (2006) reported a decrease in pH during the chemolithotrophic

nitrification process as a result of $CaCO_3$ consumption and the release of CO_2 and H^+ into the culture medium. The water hardness ($CaCO_3$) and alkalinity levels observed were above the recommended levels (4200 and 130 mg L^{-1}, respectively) (Boyd et al., 2002), and might therefore, have provided the physiological conditions for growth and survival of organism. Adequate concentrations allowed shedding and proper formation of the exoskeleton, promoting growth and survival (McGraw and Scarpa, 2003).

Concentrations of TAN and nitrite, recorded during the experiment, were maintained at adequate levels as recommended for juveniles of Pacific white shrimp (Lin and Chen, 2001, 2003). The low concentrations of nitrite, observed during the culture period, suggest the complete oxidation of ammonia to nitrate (Cohen *et al*., 2005). Studies evaluating water quality in zero-exchange systems report low concentrations of ammonia and nitrite (Burford *et al.,* 2004; McIntosh *et al*., 2000; Ray *et al.,* 2010; Vinatea *et al*., 2010; Wasielesky *et al.,* 2006), resulting from the removal of these compounds by microbial community (Ebeling *et al*., 2006)

The relationship between adding carbohydrates, reducing ammonium and producing microbial proteins depends on the microbial conversion coefficient, the C/N ratio in the microbial biomass and the carbon contents of the added material (Avnimelech, 1999). Ammonia concentration and feed protein have a direct proportional relation in aquaculture systems (Li and Lovell, 1992; Hari *et al*., 2004_a, 2006). Accumulation of dissolved nitrogen, especially ammonium, as a result of food addition and excretion of organisms reared at high density, are the main problems in intensive shrimp culture systems, affecting their food ingestion, growth and survival rates. Avnimelech (1999, 2009) with the help of a series of experiments, proved that addition of carbohydrate reduces the need of dietary protein concentration and also decrease the TAN level in the system. In this experiment, the wheat flour effectively reduced the TAN level followed by tapioca flour.

The presence of NO_2-N and NO_3-N in both control and BFT treatments indicates the occurrence of nitrification processes in the culture systems. While NO_2-N concentration in BFT treatments seems to be relatively

stable, the opposite was observed in the control which could be explained by the higher rate of nitrification processes in these treatments. For the first 60 days of the experimental period, NO_3-N accumulation was observed in all the biofloc treatments and control which were followed by a sharp decline on 75th day. This decrease probably relates to NO_3-N uptake by microbes in the treatments in particular when there was limited ammonia-nitrogen available in the water (Hargreaves 1998). As most of the ammonia in the culture system is up taken by heterotrophic bacteria, the availability of NO_3-N in BFT system thus allows the phytoplankton to grow (Kirchman 1994; Middelburg & Nieuwenhuize 2000). Nitrogenous constituents were at safe levels for [ammonia (Frias-Espericueta *et al.,* 1999; Khun *et al.,* 2010), nitrite (Lin and Chen, 2003), and nitrate (Wickins, 1976)] in the biofloc treatments and control which are suitable for shrimp culture.

The average TSS levels were recorded to be 158, 285, 493.5 and 485 mg L^{-1} in the control, BFT_S, BFT_T and BFT_W, respectively. The TSS observed in the present study is within the recommended level of <500 mg L^{-1} for penaeid shrimps (Samocha *et al*., 2007) The average levels of VSS were recorded 282, 427, 494 and 526 mg L^{-1} in the control, BFT_S, BFT_T and BFT_W, respectively. Several authors have indicated that a similar trend of concentration of TSS and VSS was observed which is beneficial to the shrimp and to the system stability (Ebeling *et al*., 2006; Cohen *et al.,* 2005; Schryver *et al*., 2008; Baloi *et al.,* 2013). The EC value was recorded in the range of 31.60 to 46.73 mS cm^{-1} which is well suitable range for shrimp aquaculture (Boyd *et al*., 2002; Araneda *et al*., 2008).

The average concentration of chlorophyll a 283.4, 439.14, 516.77 and 553.67 µg L^{-1} were recorded in the control, BFT_S, BFT_T and BFT_W, respectively. The decrease in the concentrations of chlorophyll a during the study is probably associated with the use of carbon sources to control ammonia. The use of carbon sources in biofloc systems promotes succession and dominance of bacteria over microalgae (Gonzalez-Felix *et al.,* 2007; Ju *et al.,* 2008). Chlorophyll a concetrations in the present study was lower than those reported (Burford *et al.,* 2003; 2004, Decamp *et al.,* 2007; Martinez-

Cordonova *et al.*, 2002) for pacific white shrimp production in zero-water exchange systems. The biochemical oxygen demand (BOD), potentially high in the biofloc tanks due to increased level of microbial density led to decreased oxygen availability for shrimp (Azim and Little, 2008).

Floc volume levels increased gradually during the experiment period and the fluctuation over the time was basically consistent. After 90 days, average floc volume levels in both biofloc treatments were 15.5, 32.1, 33.6 and 43.0 ml L^{-1} in the control, $BFT_{S,}$ BFT_T and BFT_W respectively. The recorded level of floc volume was sufficient for the growth of shrimp (Avnimelec, 2012). The total heterotrophic bacterial count in the BFT_w, was higher than that reported (Burford *et al.,* 2003). Burford *et al.* (2004) reported that the plankton density in the zero water exchange system was 10^4 cells ml^{-1}. The plankton density was very low in all the treatments due to the limited light and the presence of carbon source. The application of carbon source enhances the microbial growth rather than microalgae.

5.3. Plankton Composition

Zooplankton was highly abundant throughout the study in all the biofloc treatment tanks. Nematodes, ciliates and copepods were present in low abundance at the beginning of the study and became less due to grazing by the shrimp. Nematodes and copepods were seen almost exclusively grazing on and within the particles and cyanobacteria were also principally located within biofloc particles. Shrimp might have consumed a portion of the zooplankton community. Previous authors have shown that these nutritious plankton are an important food item for shrimp (Moss *et al.,* 2001; Sullivan and Moncreiff, 1990). These zooplankton ingest algae and bacteria, suggesting that a change in the abundance of these primary consumers could have a top-down trophic effect. However, the two zooplankton groups are thought to be nutritious prey for shrimp. In terms of potential nutrition, a decrease in the abundance of zooplankton might not be desirable for the shrimp. Phytoplankton abundance was limited in all the biofloc treatments due to the light limitation and carbon source application. The similar types of plankton community were observed by Ray *et al*. (2010).

5.4. Growth Parameters

In the present study, the growth (in terms of final weight, weight gain and specific growth rate) of the shrimp in both the biofloc treatments was significantly higher than that obtained in the control, while the feed conversion rate was significantly favourable. The FCR of 1.03 obtained in the treatment BFT_W was at the lowest and the highest FCR was 1.69 in control. The FCR values in BFT are lesser than that reported (1.2 to 2.5 for successful intensive shrimp production (Venero *et al*., 2009). Growth rate and final individual shrimp weight were significantly higher in the treatment BFT_W. Although no significant differences were observed on survival but there were significant differences in FCR, SGR, PER, FER between BFT_W, BFT_S, BFT_T and control treatments, indicating that the appropriate quantity of carbohydrate addition was helpful in good growth and survival of *L. vannamei.* These results are in conformity with earlier findings that the growth rate and feed utilization improved in *L. vannamei* (Wasielesky *et al*., 2006), *P. monodon* (Arnold *et al*., 2009) and *P. semisulcatus* (Megahed, 2010), cultured with bioflocs system. These might be due to the synergistic effects of the improved water quality, higher bacterial and zooplankton densities. Studies have indicated that carbohydrate addition can result in the production and accumulation of bio-flocs (Avnimelech, 2007; Emerenciano *et al*., 2011; Gao *et al*., 2012), which could serve as an important food source for the zooplankton and thus could increase the growth of the shrimp. It has been demonstrated that zooplankton serve as a supplemental food source for *L. vannamei* (Anderson *et al*., 1987; Chen and Chen, 1992), and therefore could increase the conversion efficiency of microbial protein into *L. vannamei* protein. Studies have also demonstrated that bio-flocs serve as a good source of protein for shrimp and lower the demand for feed protein (Burford and Lorenzen, 2004; Tacon *et al*., 2002). Avnimelech *et al.* (2008) also demonstrated that carbohydrate addition can lead to increase in protein utilization and supply of essential lipids and vitamins for the growth of shrimp. Hari *et al.* (2004; 2006) demonstrated that carbohydrate addition could replace the high protein diet into low protein diet for intensive culture system.

5.5. Proximate Composition of Shrimp

The proximate values of shrimp are directly proportional to the biofloc proximate values. It is confirmed that presence of high nutritional composition in the biofloc resulted in the growth of shrimp. The body composition of *L. vannamei* was similar to the observation made by Xu and Pan (2012).

5.6. Nutritional quality of biofloc

The proximate composition, energy contents and C:N ratio of biofloc, derived from biofloc tanks fed with different carbon sources were significantly different ($P<0.05$) with regarded to all the nutritional parameters of biofloc between the biofloc treatments and control, indicating that the biofloc quality was not related to the quality of the feeds but varied according to carbon source. In this study, the higher protein content was observed in the biofloc produced in the treatment BFT_W (53.65%) followed by BFT_T (52.03%) and BFT_S (45.98%). Biofloc that contains more than 50% crude protein, 4% fibre, 7% ash and 22 kJ g^{-1} energy on dry matter basis can be considered as appropriate in fish nutrition especially for herbivorous/omnivorous fish species (Webster and Lim, 2002). It was confirmed that the biofloc produced in this study provided good nutrition to the shrimp. Proximate compositions of the bioflocs from the current experiment reveals that they contained appropriate crude protein and crude lipid for shrimp nutrition for at least omnivorous *L. vannamei* (Cuzon et al., 2004).The biofloc was able to provide sufficient mineral content for the growth of shrimp. The mineral composition (Fe, Zn, Ca, Mg, Na, K and total phosphorus) observed in the biofloc were similar to the mineral composition observed by Khun *et al.* (2010). The higher mineral level was observed in the biofloc produced in the treatment BFT_W followed by BFT_T and BFT_S.

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SUMMARY

The aquaculture is the fastest growing industry with a rate of 9% per year since the 1970s. However, this industry has come under scrutiny for contribution to environmental degradation and pollution. As a result, requirement for more ecologically sound management and culture practices remains necessary. Moreover, the expansion of aquaculture is also restricted due to high cost of land its strong dependence on fishmeal and fish oil for feed preparation. These ingredients are one of the prime constituents of feed for commercial aquaculture. Feed costs represent at least 50% of the total aquaculture production costs, which is predominantly due to the cost of protein component in commercial diets. The Pacific white shrimp, *L. vannamei* is an important shrimp in aquaculture of Western hemisphere. It is expensive and available throughout the year. It is a hardy species having good flavour and taste. It has equal nutritional value as compared to other animal food products. It is a voracious feeder and mostly feeds on organic detritus and benthos. *L. vannamei* is farmed and is well established in several countries in East, Southern hemisphere and South Asia and plays significant role in shrimp aquaculture production. This species is one of the candidate species for aquaculture. Farming *L. vannamei* is generally conducted extensively in grow-out ponds, and has been developed as an indoor high-intensive farming system to meet growing world demand. Interest in closed aquaculture systems is increasing, mostly due to biosecurity, environmental and marketing advantages over conventional extensive and semi-intensive systems. When water is reused, some risks such as pathogen introduction, escapement of exotic species and discharging of waste water (pollution) are reduced and even eliminated. Such environmental issues have created a large demand for productive, efficient and sustainable shrimp farming systems that have low impact on the environment and are more likely to be disease free.

The environment friendly aquaculture system called “Biofloc Technology (BFT)” is considered as an efficient alternative system since nutrients could be continuously recycled and reused. This technology is not popular in

India. Therefore, the study envisages feeding microbial biofloc to *L. vannamei* formed by using low cost carbohydrate material as a carbon source for the production of biofloc which boost the shrimp production by improving the conversion of nutrients into harvestable products. The sustainable approach of such system is based on growth of microorganism in the culture medium, benefited by the minimum or zero water exchange.

The study was conducted to evaluate and compare the effects of microbial bioflocs on water quality, changes in microbial diversity and growth performance of the pacific white shrimp (*L. vannamei*), fed with different carbon sources. The sampling was carried out at fortnightly interval to investigate the growth, microbial and water quality parameters of biofloc system. In the experimental set-up there were three biofloc treatments and one control, managed in 500 L indoor tanks i.e. biofloc tank fed with sugarcane molasses (BFT_S), BFT fed with tapioca flour (BFT_T) BFT fed with wheat flour (BFT_W) and control without biofloc. The postlarvae of *L. vannamei* with an average body weight of 0.15 ± 0.02 g were stocked at the rate of 130 PL m^{-2} and each biofloc treatment and the control was tested in triplicate over a period of 90 days. Commercially available pelleted shrimp feed was applied at 1.5% of the total shrimp biomass daily in each experimental tank.

Water quality parameters like temperature, salinity, alkalinity, pH, hardness, dissolved oxygen, total ammonia-N, nitrite-N, nitrate- N, total suspended solids, volatile suspended solids, Ec, chlorophyll a, biological oxygen demand, Floc volume, total heterotrophic bacteria count and plankton were analysed and found within the optimum range in biofloc treatments. The addition of carbohydrate significantly ($P<0.05$) reduced the total ammonia nitrogen (TAN), nitrite and nitrate in the biofloc treatments and also between the biofloc treatment and control. It significantly ($P<0.05$) increased the total heterotrophic bacterial (THB) population in the biofloc treatment. Body weight of shrimp was recorded at the interval of 15 days to assess the growth performance. The proximate

composition of the feed revealed that the nutrient content were as per the requirement of *L. vannamei.*

Growth parameters like average body weight, growth rate, specific growth rate, feed conversion ratio, protein efficiency ratio, feed efficiency ratio, total weight, and total weight gain efficiency ratio were significantly different between the biofloc treatments and control. There was a significant difference ($P<0.05$) between treatment and control group in terms of final average body weight (ABW) at harvest confirming the utilization of biofloc by shrimp as a food source. The FCR differed significantly between biofloc treatment and control ($P<0.05$). The shrimp survival was not affected ($P>0.05$) by the biofloc treatments, ranging between 82.2% and 90.3%. The proximate composition of the biofloc and shrimp were differed significantly between the biofloc treatments and control. The higher protein content was found in the biofloc derived from BFT_W. There were significant differences (<0.05) in mineral composition such as Fe, Zn, Ca, Mg Na, K, and total phosphorus between the biofloc treatments and the control. The higher mineral content was found in the biofloc treatments than control.

The taxonomic compositions of organisms in the bioflocs were identified tintinids, ciliates, copepods cyanobacteria, and nematodes. Among the groups of organism nematodes were the most dominant groups next to the bacteria in all the biofloc treatment tanks. The total numbers of organisms were significantly higher in the wheat flour fed biofloc tanks followed by the tapioca fed biofloc tanks and control. DGGE analysis with PCR-amplified 16s rRNA, gene using universal DGGE primers (F968 and R1378) and further sequencing of excised band obtained from polyacrylamide gel, revealed the nearest identity of 27 microorganisms from the biofloc treatments, 14 microorganisms from BFT_S, 13 from BFT_T, 16 from BFT_W and 5 from control. From sequence analyses *Ruegeria* sp. *Chitinophaga pinensis, Roseobacter litoralis, Hyphomonas neptunium* and *Phaeobacter gallaeciensis* were found to be the predominant bacteria present in the biofloc treatments.

Based on the results of the present study it can be concluded that,

- There is a direct link between the addition of carbohydrate and improved growth of shrimp as inorganic nitrogen species in the water column is reduced due to uptake by greater number of heterotrophic bacteria, making a system more sustainable. The BFT_W was more effective in reducing inorganic nitrogen species than tapioca flour and sugarcane molasses. So from this study it is recommended that wheat flour could be used effectively for the biofloc production and also to achieve higher production of shrimp.
- Biofloc clearly contributed to the growth and production of shrimp cultured in biofloc systems.
- The nutritional quality of biofloc was appropriate at least for omnivorous shrimp species including *L. vannamei*
- It can be suggested on the basis of preliminary molecular results of the present study, that seawater based BFT needs further exploration using molecular tools.

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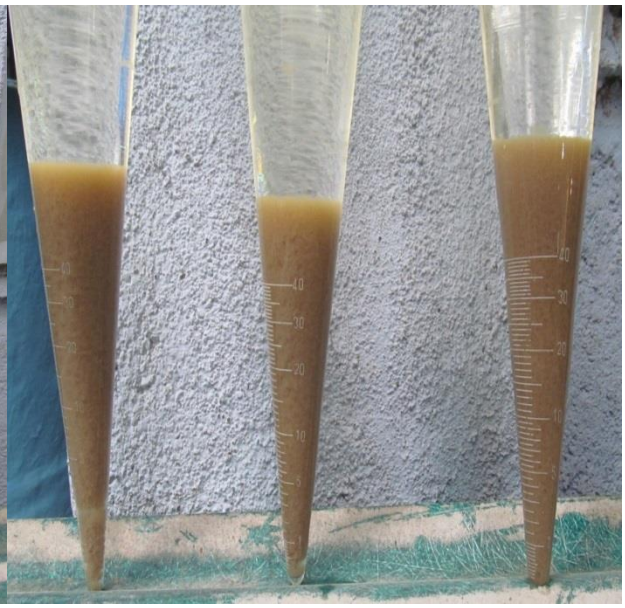
PLATES

Biofloc production

Feeding to the shrimp



Measurement of Floc density (ml L^{-1})

Plate 1. Biofloc production and measurement

BFT_S-Sugarcane Molasses

BFT_T- Tapioca Flour

BFT_W-Wheat flour

Control

Plate 2. Harvested research animals from the treatment and control tank

APPENDIX I

MEDIA USED

1) 0% Denaturant Solution

	8% Gel	10% Gel
40% Acrylamide/Bis	20 ml	25 ml
50X TAE buffer	2 ml	2 ml
Double Distilled water	78 ml	73 ml
Total volume	100 ml	100 ml

2) 100% Denaturant Solution

40% Acrylamide/Bis	20 ml	25 ml
50X TAE buffer	2 ml	2 ml
Formamide	40 ml	40 ml
Urea	42 g	42 g

3) 40% Acrylamide/Bis

40% Acrylamide/Bis	38.93 g
Bis-acrylamide	1.07 g
Double Distilled water	to 100 ml

4) 50X TAE

Tris base (2 M)	242.0 g
Glacial acetic acid (1M)	1 M
0.5 M EDTA (pH 8.0)	100 ml
Double distilled water	to 1000 ml

5) Nutrient Agar

Peptone	5.0 g
Sodium chloride	5.0 g
Beef extract	1.5 g
Yeast extract	1.5 g
Agar	15.0 g
Final pH (at 25°C)	7.4±0.2

Suspend 28 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Dispense as desired and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mix well before pouring.

APPENDIX II

%	Percentage
µg	Microgram
‰	Parts per thousand
ANOVA	Analysis of Variance
AST	Activated suspension technique
BFT	Biofloc technology
BLAST	Basic local alignment search tool
BOD	Biochemical oxygen demand
C	Carbon
C: N	Carbon nitrogen ratio
CF	Crude Fiber
CFU	Colony forming unit
CP	Crude Protein
DGGE	Denaturing gradient gel electrophoresis
DM	Dry Matter
DNA	Deoxyribo Nucleic Acid
DO	Dissolved Oxygen
EE	Ether Extract
FAO	Food and Agriculture Organisation
FCR	Feed Conversion Ratio
Fig	Figure
FISH	Fluorescence In Situ Hybridization
FRP	Fibre-Reinforced Plastic
g m^{-3}	Gram per meter cube
g or rpm	Revolution per minute
g	Gram
h	Hour
HDPE	High-density polyethylene
Kcal	Kilocalorie
Kg	Kilogram

M	Meter
MBR	Membrane biological reactor
mg	Milligram
mM	Millimolar
mt	Million tonnes
N m^{-3}	Nitrogen per meter cube
n mole	Nano mole
N	Normality or normal
NFE	Nitrogen Free Extract
PCR	Polymerase chain reaction
PER	Protein Efficiency Ratio
PHA	Poly hydroxyl alkanoate
PHB	Poly-β-hydroxybutyrate
ppm	Parts per million
RNA	Ribo Nucleic Acid
SE	Standard Error
SGR	Specific Growth Rate
TC	Total Carbohydrate
U	Units
wt	Weight
µl	Microlitre
µM	Micromole
∑	Summation
ng g^{-1}	Nanogram per litre
mg l^{-1}	Milligram per litre
min	Minute