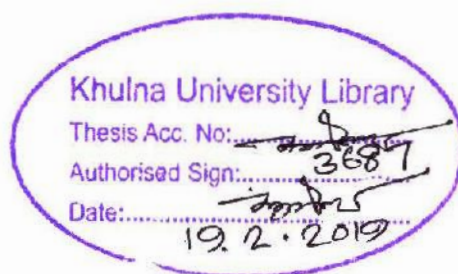


**Effect of different C/N ratios on volume and potential microbial
composition of flocs in prawn culture system**



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composition of flocs in prawn culture system**

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By

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Declaration

Effect of different C/N ratios on volume and potential microbial composition of flocs in prawn culture system

The results submitted in this thesis are entirely of the candidate's own investigations and no part of the results has been accepted for any degree, nor is it being concurrently submitted for any degree.

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DEDICATED TO

My Beloved Parents

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Contents

Acknowledgement	i
List of Figures	iv
List of table	v
Abstract	vi
1 Introduction	1
1.1 Review of literature.....	3
1.1.1 Overview of aquaculture	3
1.1.2 Problems in aquaculture	4
1.1.3 Biofloc Technology	8
1.1.4 Literature gap.....	18
2 Materials and methods	19
2.1 Study area and period.....	19
2.2 Experimental Design.....	19
2.3 Development of Biofloc Technology.....	20
2.4 Laboratory Requirements for microbial assay.....	20
2.4.1 Laboratory Equipment	20
2.4.2 Reagents and chemicals	20
2.4.3 Types of media needed during culture period	20
2.5 Sample collection.....	21
2.6 Laboratory work	21
2.6.1 Floc Determination	21
2.6.2 Sterilization of equipment	21
2.6.3 Sample preparation and dilution.....	21
2.6.4 Total bacteria count.....	21
2.6.5 Determination of <i>Enterococcus</i> spp.....	22
2.6.6 Determination of <i>Clostridium</i> spp.	25
2.6.7 Determination of <i>Lactobacillus</i> spp.....	26
2.6.8 Determination of <i>Salmonella</i> spp.	26
2.6.9 Counting of colonies	26
2.7 Data analysis.....	27
2.8 Biochemical tests of bacterial composition.....	27
3 Results	28
3.1 Floc Determination	28
3.2 Total bacterial count.....	28
3.3 <i>Enterococcus</i> spp. count.....	29
3.4 <i>Clostridium</i> spp. count	30
3.5 <i>Lactobacillus</i> spp. count.....	30
3.6 <i>Salmonella</i> spp. count	31
3.7 Biochemical tests	31
4 Discussion.....	33
5 Conclusion.....	36

6	References	37
7	Appendices	47
7.1	Appendix-1	47
7.2	Appendix- 2	49

List of Figures

Figure 2.1 Layout of the experiment for <i>M. rosenbergii</i> culture using biofloc technology at different C:N ratios.	19
Figure 3.1 Volume of flocs at different C/N ratios in biofloc based prawn culture system. The error bars represent the standard deviations of nine replicates. Different letters indicate significant difference among the treatments (one-way ANOVA, $P<0.05$).	28
Figure 3.2 Total heterotrophic bacterial count (Log CFU/mL) in the flocs at different C/N ratios in biofloc based prawn culture system. The error bars represent the standard deviations of nine replicates. Different letters indicate significant difference among the treatments (one-way ANOVA, $P<0.05$).	29
Figure 3.3 <i>Enterococcus</i> spp. count (Log CFU/mL) in the flocs at different C/N ratios in biofloc based prawn culture system. The error bars represent the standard deviations of nine replicates. Different letters indicate significant difference among the treatments (one-way ANOVA, $P<0.05$).	29
Figure 3.4 <i>Clostridium</i> spp. count (LogCFU/mL) in the flocs at different C/N ratios in biofloc based prawn culture system. The error bars represent the standard deviations of nine replicates. Different letters indicate significant difference among the treatments (one-way ANOVA, $P<0.05$).	30
Figure 3.5 <i>Lactobacillus</i> spp. count (LogCFU/mL) in the flocs at different C/N ratios in biofloc based prawn culture system. The error bars represent the standard deviations of nine replicates. Different letters indicate significant difference among the treatments (one-way ANOVA, $P<0.05$)	31

List of table

Table 3.1 Results of biochemical tests for indication of specific group of bacteria.....	31
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Abstract

The use of ecofriendly method in the culture of aquatic organisms is increasing with demand as an advanced mode of aquaculture for gaining environmental sustainability. The present study was conducted by developing biofloc technology as one type of ecofriendly mode of aquaculture to evaluate the effects of C/N ratio on the volume of floc as well as on the load of several potential microbes in the floc composition. The experiment had three treatments with C/N ratios, 10, 15, and 20, namely T1, T2, T3 and a control (C). Determination of floc volume and enumeration of both some beneficial (*Enterococcus* spp., *Clostridium* spp., *Lactobacillus* spp.) and harmful (*Salmonella* spp.) bacterial load was done in the floc content of the fresh water prawn (*Macrobrachium rosenbergii*) culture tanks. Volume of floc was measured by using Cone and each of the bacteria was cultured by using specific and selective media. The results of this study demonstrated that T2 and T3 had higher floc volume compared to control C and T1 ($P<0.05$). This higher growth in C/N, 15 and 20 might be due to available carbon sources for heterotrophic bacteria to remove environmental nitrogen given by feed supplements. In addition, the loads of *Enterococcus* spp. was found to be highest in T2, followed by T1 and C, and T3 ($P<0.05$). Lower *Enterococcus* spp. load in T3 might be due to high organic load that might deteriorate the environmental quality for this bacterial growth. However, in case of *Clostridium* spp. we did not find any different between the treatments ($P<0.05$). Moreover, higher *Lactobacillus* spp. load was in T1 and T2 compared to T3 and C, which indicates that growth of *Lactobacillus* spp. require a fair range of carbon source, and lower and higher carbon load might be insufficient and/ or toxic for their growth. Finally, presence of no *Salmonella* spp. indicates a healthy culture system without any contamination, and/ or might be due to competitive exclusion or inhibitory activities of beneficial bacteria against pathogenic bacteria. In conclusion, biofloc technology might be a good approach of sustainable prawn aquaculture through application of proper C/N ratios and further research should be conducted for more advance biofloc based aquaculture.

Chapter One

INTRODUCTION



1 Introduction

Aquaculture is a worldwide activity and world aquaculture has grown tremendously during the last decades and becoming an economically important industry (Subasinghe *et al.*, 2009). Through the use of intensive aquaculture, production of both freshwater and marine food fish has been increased significantly. However, the aquaculture industry has come under scrutiny for contribution to environmental pollution and degradation. Therefore, it is now important to adapt with environment friendly sustainable management practices in aquaculture industry. Development of biofloc technology in aquaculture system might be one of the eco-friendly technologies and an alternative protein source. Biofloc Technology (BFT) is considered as an efficient alternative system since nutrients could be continuously recycled and reused. The sustainable approach of such system is based on growth of microorganism in the culture medium, benefited by the minimum or zero water exchange (Crab *et al.*, 2009).

Bioflocs are macroaggregates (flocs) of bacteria, algae, protozoa (also known as zooplankton) and particulate organic matter such as uneaten food and feces. The flocs are held together by a loose matrix of mucus secreted by the bacteria, bound by filamentous microorganisms or held together by electrostatic attraction (Hargreaves, 2013).

In BFT, microorganisms present a key role in nutrition of cultured animals. The macroaggregates is a rich protein-lipid natural source available “*in situ*” 24 hours per day (Avnimelech, 2007). The consumption of biofloc by shrimp or fish has demonstrated innumerable benefits such as improvement of growth rate, decrease of FCR and associated costs in feed (Burford *et al.*, 2004). Regarding to maintenance of water quality, control of bacterial community over autotrophic microorganisms is achieved using a high carbon to nitrogen ratio (C:N) (Avnimelech *et al.*, 1994).

The excretion of nitrogenous metabolic wastes and their assimilation by heterotrophic bacteria maintain a balance by manipulating the carbon-to-nitrogen ration (C:N ratio) by the addition of carbon sources in the water. High carbon to nitrogen ratio is required to guarantee optimum heterotrophic bacteria growth (Avnimelech, 2007).

Cheap sources of carbohydrates such as molasses, glycerol and plant meals (i.e. wheat, corn, rice, tapioca, etc) are applied to manipulate the Carbon and Nitrogen (C/N) ratio. This

manipulation stimulates heterotrophic bacterial growth as well as controls inorganic nitrogen concentration in the system through assimilation of ammonia into bacteria as single-cell microbial protein (Taw, 2010). The microbial protein would then be eaten by the fish, thereby recycling proteins that are excreted from the fish. As only 20-25% of fed protein is retained in the fishes raised in intensive system, the remainder are excreted into the system as ammonia and organic nitrogen in feces and feed residues (Avnimelech, 1994).

The biofloc (microorganisms) has two major roles: (i) maintains water quality by the uptake of nitrogenous compounds to generate microbial proteins on-site; and (ii) increases culture feasibility by reducing feed conversion ratio through higher protein utilization and lower inputs of commercial feed, hence decreasing feed cost (Schryver *et al.*, 2008).

In the present study, the biofloc technology was adopted into the prawn culture system by using molasses and after those different types of important flocs that are essential for prawn growth was determined. In the biofloc the aggregation of several bacteria has also effects on the better growth and development of aquatic species (Avnimelech *et al.*, 1994). So, the abundance of several potential beneficial and harmful bacteria was also enumerated from the aggregated flocs of the prawn culture system.

Objectives of the Research:

The main objectives of the present study were –

- To determine the volume of flocs in the biofloc treated prawn culture system using different C:N ratios.
- To enumerate the potential microbial composition (*Enterococcus* spp., *Clostridium* spp., *Lactobacillus* spp. and *Salmonella* spp.) in the developed flocs.

1.1 Review of literature

1.1.1 Overview of aquaculture

Within inland water total production of captured and farmed aquatic animals, aquaculture consolidated its dominating share from 68.6% in 2000 to 81% in 2015. World aquaculture production of fish and plants combined reached 106 million tonnes in live weight in the year 2015, with a total estimated first-sale value of US\$163 billion. By production volume, the sub-sector of aquaculture of aquatic animals has been dominated by finfish farming, which represents the major aquaculture product in many countries. The contribution of finfish has been maintained between 63-68% in the last two decades. Crustacean farming improved its share from less than 5% before 2000 to close to 10% in the past decade (FAO, 2016).

Bangladesh is considered one of the most suitable regions for fisheries in the world, with the world's largest flooded wetland and the third largest aquatic biodiversity in Asia after China and India. Bangladesh puts out about 2.0 million metric tons of aquaculture produce annually. Shrimp culture leads aquaculture farming in Bangladesh. This increase in the aquaculture production has been made possible with the implementation of scientific and technological modernization. From 2000 to 2016, aquaculture production increased from 712,640 and 2,060,408 metric tonnes, a much larger quantity than wild capture production (1.023 million t) in 2016. There has also been a recent increase in the value of fishery exports, with more than US \$34.08 billion in 2010 rising to more than US \$46.60 billion in 2015. However, fisheries production is well below production targets despite the large gains seen in the aquaculture sector (Shamsuzzaman *et al.*, 2017).

Carp polyculture at the individual small holder level has the greatest potential for expansion since it can, through the implementation of more intensive culture systems including the application of fertilizers, use of supplemental feeding and improved management practices (Gupta *et al.*, 1999). At present annual average fish production using pond culture is 2 609 kg/ha (DoF, 2005).

Shrimp farming is a capital intensive business with total production costs of US\$ 735 per ha/crop for extensive system, US\$ 1837 per ha/crop for improved traditional systems and US\$ 9184 per

ha/crop for semi intensive systems, the corresponding net income however is US\$ 1 275, US\$ 2 204 and US\$ 153 061 per ha/crop respectively (ICLARM, 2002).

Fishery accounted for 3.8% of whole Bangladesh GDP, as 22.76% of total GDP in agriculture. In 2011 - 2012, its all quantity of aquatic products was 3.26 million tons, and the aquatic products exported foreign exchange earning accounted for 2.46% of total foreign exchange receipt in the whole nation. Not only that, fishery industry also provided about 60% animal protein consumption of the country, and directly or indirectly supplied aquaculture industry related work for almost 11% population of Bangladesh (Shamsuzzaman *et al.*, 2017).

This increase in the aquaculture production has been made possible with the implementation of scientific and technological modernization. Fish contribute a significant amount of animal protein to the diets of people in Bangladesh, about 63% of which comes from aquatic animals. In Bangladesh, fish is mainly derived from two sources: capture and culture. Aquaculture has shown tremendous growth in the last two decades, exhibiting by about 10% average annual growth in production. Capture fisheries, although still the major source of supply of fish, have become static or are in decline due to over-fishing and environmental degradation, and it is now believed that aquaculture has the greatest potential to meet the growing demand for fish from the increasing population. At present, aquaculture production accounts for about one-third of the total fish production in Bangladesh (E-Jahan *et al.*, 2017).

1.1.2 Problems in aquaculture

Aquaculture creates several problems as well there have several problems to adopt an aquaculture programme. Generally, it can be noticed that, water color turns to deep green because of the presence of excessive algae in water, which is the result of overabundance of nutrient like phosphorus, particularly when the water is warm and the weather is calm. Even heavy fish loads contribute to green pond water because fish waste is broken down by beneficial bacteria through a series of steps into nitrate, which is food for plants and algae. As per the World Health Organization (WHO) standard when the population of algal cells exceeds 100,000 cells/ ml, the condition is termed as algal blooms. Problem of blooms are generally occur in the summer months. In general, the major negative effects of algae occur indirectly. Excessive algae growth can lead to a lack of dissolved oxygen in the water,

particularly, dissolved oxygen content drops during night (due to excessive consumption of O_2 and absence of photosynthesis). In this situation, due to the heavy shortage of dissolved oxygen, fish start grasping on the water surface. The appearance of muddy water in farm ponds may be the result of physical, environmental, or biological factors. Muddy water can have negative effects other than detracting from the aesthetics of the pond (Hussan *et al.*, 2016).

Oxygen depletion in pond and fish grasping for air is a commonly encounter problem by the fish farmers. Generally during very early in the morning, fish start floating and grasping on the water surface. It happens due to the shortage of dissolved oxygen in water. If shortage of oxygen is very high and long lasting, then fish start to die and dead fish have their mouths wide open. Large fish may die first, but all sizes of fish are usually affected. Mollusks and crustaceans gather at the pond edges. The causes behind low dissolved oxygen content in pond water may be: dense plankton blooms or dense stands of pond weeds, several days of cloudy weather that reduces plant oxygen production, high temperatures, which decrease the solubility of oxygen in water and increase oxygen consumption by plants and animals, sudden die-off of plants or algae, especially associated with herbicide use, unusual weather patterns such as storm fronts and heavy cold rain, overstocking of fish, excessive fertilization, or high feeding rates, decomposition of organic matter, leaves and branches of trees in water, excessive application of wet cow dung in pond, excessive turbidity in water, high iron content in soil (Ghose, 2014).

In ponds, ammonia is produced by excretion of fishes, crustaceans; microbial decomposition of organic materials; urea and ammonium fertilizers; supplementary feed etc. Unionized ammonia fraction is more toxic to fish. A concentration of 0.02-0.05 mg/L of unionized ammonia is safe for fishes. The concentration of unionized ammonia increases with increasing pH and temperature and at higher concentration it is highly toxic to fishes. Besides pH and temperature, some other parameters are also having indirect effects on the ammonia toxicity. Ammonia is more toxic when dissolved oxygen concentration in fish ponds is low; toxicity of ammonia decreases with low pH and increased CO_2 concentration in fish ponds; toxicity of ammonia decreases slightly by high concentration of calcium (Mook *et al.*, 2012).

Freshwater fish-ponds should be freed from hydrogen sulphide (H_2S). Any detectable concentration of H_2S in water creates stress to fish. Hydrogen sulphide is produced by chemical reduction of organic matter that accumulates and forms a thick layer of organic deposit at the

bottom. Unionized hydrogen sulphide is toxic to fish. Due to buildup of extra feed and organic matter at the pond bottom, soil of the bottom turns to dark and smelly. Particularly, the problem can be severe in a pond where fish culture is going on for long time. As a result, fish may face mass mortality due to the toxic gases at the bottom (Hussan *et al.*, 2016).

1.1.2.1 Diseases in aquaculture

Outbreak of disease is more common in farming operations than the wild as a result of higher levels of stress in fish, high stocking densities and establishment of conditions conducive to incubation of disease organisms. Aquaculture provides opportunity for amplification of disease, though notably it also facilitates early detection of outbreaks due to frequency of testing to protect valuable fish stocks. Additionally, increased food resources near farm cages attract large concentrations of escaped and wild fishes, which may act as vectors for the transfer of disease and parasites to other native fish (Carss, 1990). Disease problems constitute the largest single cause of economic losses in aquaculture. Bacterial infections constitute the most important source of disease problems in all the various types of production. Gram-negative bacteria cause epizootics in nearly all cultured species (Austin *et al.*, 2012).

Aquaculture operations can spread parasites and disease into the wild. Just like commercial chicken coops must be kept clean and are notorious for the spread of disease, farmed fish and shellfish are subject to the same circumstances. Also, farmed fish have an increased chance of getting parasites such as sea lice, as opposed to fish that live and breed in their natural environment (Woo, 1992).

Fungal diseases constitute the second most important source of losses, especially in the culture of crustaceans and salmon. External protozoan parasites are responsible for the loss of large numbers of fry and fingerling fin fishes and are a cause of epizootics among young shellfish. The number of therapeutants approved by the Food and Drug Administration is limited. Research to support the registration of promising therapeutic agents is urgently needed (Meyer, F. P., 1991). Farmed fish are also exposed to disease through the use of unprocessed fish used as a food source, as opposed to safer processed fish pellets (Bryan, 1977).

The major disease problems that occur in aquaculture ponds are parasitic, fungal, and bacterial in origin. Important disease problems that generally encounters are argulosis, lerneasis, protozoan

diseases caused by *Trichodina*, *Costia*, *Ichthyobodo*, Myxosporidean diseases, gill flukes such as *Dactylogyrus*, and *Gyrodactylus*; fungal diseases such as saprolegniasis, branchiomycosis, Epizootic Ulcerative Syndrome; bacterial diseases such as aeromoniasis (fin and tail rot)/red disease, edwardsiellosis, *Columnaris* and vibriosis etc. Besides these, an algal bloom is a major problem that depletes the dissolved oxygen in the ponds (Reno, 1998).

1.1.2.2 Environmental problem

Like a giant aquarium, land-based fish farms live in tanks containing dirty water that must be changed. Depending on the system's set-up, this can result in the discharge of significant amounts of wastewater containing faeces, nutrients and chemicals released into the environment. The release of this matter can result in algae blooms which eventually remove dissolved oxygen in the receiving waterway, or eutrophication. Zero oxygen content results in deadly fish kills (Camargo, 2006). Additionally, chemicals such as antibiotics and water treatment agents are commonly used in the aquaculture industry and aquaculture systems need to be closed, or wastewater treated prior to discharge (Cabello, 2006).

Fish farming operations result in the release of a number of wastes into the aquatic environment. These include uneaten fish food, fish excretory products and organic matter from net-cleaning that enter the water column and/or settle to the seabed. The major components of solid and dissolved waste are various forms of carbon, nitrogen and phosphorous (EAO, 1998; Ritz & Lewis, 1989). The effects on the food chain from this additional organic input are many and varied, the input leading to water column nutrient enrichment and accumulation of organic matter in the sediments. In the water column, soluble nutrients can alter the species composition and density of phytoplankton, increasing the risk of toxic algal blooms (DPIF, 1997). The accumulation of organic matter on the seabed, especially in areas of poor current flow, can produce major changes in the sediment chemistry. Changes typically associated with severe organic enrichment are a reduction in sediment oxygen levels and the subsequent production and release of methane and toxic hydrogen sulphide (Pearson and Rosenberg, 1979).

Chemicals are used in finfish aquaculture for a wide range of applications. Chemicals are not only used in fish health, but also to control nuisance organisms on equipment such as nets, and to disinfect and improve water quality (NPI, 2001). The use of such chemicals raises a number of

environmental concerns, and they must be registered with the National Registration Authority before use (NPI, 2001).

The negative impacts of the “biological contamination” for the introduction of exotic aquacultural species on the native populations have been well documented. The main reported problems are the displacement of native species, competition for space and food, and pathogens spread (Naylor *et al.*, 2000).

1.1.2.3 Feed problem

Aquaculture can also negatively impact the environment through overfeeding fish. In addition, feed can also account for up to 60% of the total production costs in commercial aquaculture. Aquaculture feed management is absolutely vital because the schedule that fish farmers follow to feed their fish is directly correlated to economic and environmental sustainability (White, 2013). The universal and primary concern among aquaculturists is finding a way to distribute feeds that meet the nutritional standards of the fish at ration sizes that promote both growth and proper feed conversion ratio (FCR) (White, 2013).

Although aquaculture has been proclaimed as a solution to avoid overfishing, it has contributed in more or less proportion to the fisheries collapse. Fishermen who work in places near to aquaculture farms argue that the contamination produced by farms has decreased the population of aquatic organisms and in consequence their volume captures. Additionally, another problem of similar magnitude is the extremely high aquaculture's dependence of fishmeal and fish oil, which could be another non-sustainable practice in aquaculture. The proportion of fishmeal supplies used for fish production have increased from 10% in 1988 to more than 30% in the last years, which classifies aquaculture as a potential promoter of the collapse of fisheries stocks worldwide (Avnimelech, 2009).

1.1.3 Biofloc Technology

As the human population continues to grow, food production industries such as aquaculture will need to expand as well. In order to preserve the environment and the natural resources, this expansion will need to take place in a sustainable way. Biofloc technology is a technique of enhancing water quality in aquaculture through balancing carbon and nitrogen in the system. The

technology has recently gained attention as a sustainable method to control water quality, with the added value of producing proteinaceous feed in situ (Crab *et al.*, 2012).

In aquaculture, the “biofloc” system acts like a retention trap for the nutrients in the pond, and reduces maintenance costs because it can be used as food supplement for the commercial organisms being cultured, which provides an added value by improving the food consumption rate (Azim & Little, 2008). The biofloc system is developed under the same principle that regular waste water treatment plants have, in which the microbes grow from feces of the cultured organisms being, transforming it into less complex organic products that can be consumed by other organisms and return to the food chain (Avnimelech & Kochba, 2009). Biofloc technology demands regular carbohydrate addition and additional aeration expenses to keep biofloc in suspension and for microbial respiration. Carbohydrate is added by maintaining a ratio with the released nitrogen in the aquatic environment. The additional carbohydrate acts as the substrate for better development of several microbial communities in the culture area. At high C:N ratio, microbial community within the culture system converts inorganic nitrogen metabolites into biofloc (Avnimelech, 2007; Crab *et al.*, 2012).

1.1.3.1 Structure of Floes

The term “biofloc” applies to a compound made out of 60 to 70% of organic matter, which includes a heterogeneous mixture of microorganisms (fungus, algae, protozoans, and rotifers) and of 30 – 40% of inorganic matter such as colloids, organic polymers, and dead cells. They can reach a size up to 1000 μm , irregular shape, full of pores, and allow the pass of fluids (Chu & Lee, 2004). To establish the BFT is necessary that the pond has a cover that prevents the accumulation of solid organic matter at the bottom, adding carbon sources that stimulates the growth of heterotrophic bacteria, and to keep constant aeration in the water column, which will help the combination of physical, chemical, and biological factors required for the floc formation (Azim & Little, 2008; Emerenciano *et al.*, 2011).

To achieve the establishment of heterotrophic bacteria in the biofloc, it is necessary to adjust the carbon/nitrogen (C:N) relation in the water body, and is required around 20 units of carbon to assimilate one nitrogen unit, this is obtained by adding a food of low protein and one carbohydrate such as molasses in sufficient amount (Avnimelech, 1999a; Emerenciano *com pers.*,

2011). When this rate is adequate, bacteria that grow inside of the microsystem starts to use compounds that can be toxic to the culture such as organic carbon, ammoniac nitrogen, nitrates, nitrites, and phosphates as energy sources, oxidizing them so algae, fungi, and other bacteria and filtering organisms can use them (Avnimelech, 1999a; Avnimelech, 2007). Proliferation of bacterial colonies and microorganisms generates an increase in the biofloc biomass, it increase must have a density between 10 and 15 mL so the system can keep functioning properly. The excessive can be used as source of protein for the organisms, sometimes directly or in the form of flour or feed (Avnimelech, 1999a; De Schiver *et al.*, 2008; Emerenciano *et al.*, 2011).

1.1.3.2 Biofloc in microbial community management

The addition of carbohydrate significantly increases the total heterotrophic bacterial (THB) population both in water column and sediment (Hari *et al.*, 2004). Carbon nitrogen ratio is a control element in aquaculture systems. Nitrogen control is induced by feeding bacteria with carbohydrates, and through the subsequent uptake of nitrogen from the water, by the synthesis of microbial proteins. The relationship among the addition of carbohydrates, the reduction of ammonium and the production of microbial proteins depends on the microbial conversion coefficient, the C:N ratio in the microbial biomass, and the carbon contents of the added material. The addition of carbonaceous substrate was found to reduce inorganic nitrogen in shrimp experimental tanks and in tilapia commercial-scale ponds. It was found in tilapia ponds that the produced microbial proteins are taken up by the fish. Thus, part of the feed protein is replaced and feeding costs are reduced. The addition of carbohydrates, or the equivalent reduction of proteins in the feed, can be quantitatively calculated and optimized, as shown here (Avnimelech, 1999).

Schveitzer *et al.* (2013) evaluated the effects of three concentrations of bioflocs on microbial activity, selected water quality indicators and performance of *Litopenaeus vannamei*. But the higher production of heterotrophic bacteria was found in T200 ($P < 0.05$). Regarding shrimp performance, the reduction of TSS to levels close to 200 mg L^{-1} was associated with better nutritional quality of bioflocs.

Biofloc system contains abundant number of bacteria of which cell wall consists of various components such as bacterial lipopolysaccharide, peptidoglycan and β -1, 3-glucans, and is known as stimulating nonspecific immune activity of shrimp, *Litopenaeus vannamei*. Bioflocs,

therefore, are assumed to enhance shrimp immunity because they consume the bioflocs as additional food source. Although there are benefits for having an in situ microbial community in BFT systems, better understanding on these microorganisms, in particular molecular level is needed. The presence of bioflocs in the culture medium gives positive effect on shrimp survival, growth and immune-related genes expression in *L. vannamei* postlarvae (Kim *et al.*, 2013).

The addition of carbohydrate significantly reduces the total ammonia-N (TAN), nitrite-N and nitrate-N in water and it significantly increases the total heterotrophic bacteria (THB) population in the biofloc treatments. Tintinids, ciliates, copepods, cyanobacteria and nematodes were identified in all the biofloc treatments, nematodes being the most dominant group of organisms in the biofloc. The use of wheat flour effectively enhanced the biofloc production and contributed towards better water quality which resulted in higher production of shrimp (Rajkumar *et al.*, 2015).

In 2010, Asaduzzaman *et al.* evaluated the Effects of C/N ratio and substrate addition on natural food communities in freshwater prawn monoculture ponds. An on-station trial was conducted to investigate the effects of three C/N ratios (10/1, 15/1 and 20/1) along with substrate presence or absence on natural food communities in freshwater prawn culture ponds. The biovolume of plankton, periphyton and THB increased significantly with culture time duration whereas the biovolume of benthic macro invertebrates decreased significantly with culture time indicating that freshwater prawn grazed on them. A significant interaction between C/N ratios and substrate presence or absence was only observed for plankton biovolume in the water column. The study demonstrated that plankton, periphyton and microbial biofloc communities were under-utilized by the freshwater prawn in treatment C/N20. This leaves room for increasing the stocking density of prawn and/or inclusion of periphyton grazing fish species to improve nutrient utilization efficiency and overall sustainability.

Biofloc improves water, effluent quality and growth parameters of *Penaeus vannamei* in an intensive culture system. A significant reduction in the concentration of total ammonia nitrogen (TAN) and nitrite (NO₂-N) are found in the biofloc pond than that of control pond. A significant low level of nitrogen can be found in the effluents of biofloc pond. In biofloc system, a significantly elevated heterotrophic bacterial count along with reduction in total *Vibrio* count is occurred. A significant improvement in the feed conversion efficiency (FCR) and growth

parameters of *P. vannamei* are noticed in the biofloc pond. Growth of *P. vannamei* in the biofloc pond show positive allometric pattern with an increased survival. The microbial biomass grown in biofloc consumes toxic inorganic nitrogen and converts it into useful protein, making it available for the cultured shrimp. This improved FCR and reduced the discharge of nitrogenous waste into adjacent environment, make intensive shrimp farming an eco-friendly enterprise (Kumar *et al.*, 2018).

There have potential biocontrol activity of bioflocs technology. The bioflocs do not produce growth-inhibitory substances. However, bioflocs and biofloc supernatants decrease quorum sensing regulated bioluminescence of *Vibrio harveyi*. The bioflocs have biocontrol activity against the pathogen, *Vibrio harveyi* because quorum sensing regulates virulence of vibrios towards different hosts. Interestingly, the addition of live bioflocs significantly increased the survival of gnotobiotic brine shrimp (*Artemia franciscana*) larvae challenged to *V. harveyi*. Bioflocs grown on glycerol as carbon source inhibit quorum sensing- regulated bioluminescence in *V. harveyi* and protect brine shrimp larvae from vibriosis. In addition to water quality control and in situ feed production, bioflocs technology can help in controlling bacterial infections within the aquaculture pond (Crab *et al.*, 2010).

Abbaszade *et al.* (2018) evaluated the effects of biofloc on bacterial load of water, growth performance and intestine of *Litopenaeus vannamei* with addition of spoilage date palm extract (SDE) and molasses as sugar sources. the number of total heterotrophic bacteria was increased during the study and the numbers of total heterotrophic bacteria in all biofloc treatments were higher than that of the control. The highest and the lowest numbers of fungi, *Vibrio* and *Bacillus* were found in biofloc treatment and the control treatment, respectively. The result demonstrated that the effects of SDE were better than molasses, which could be due to the presence of useful compounds such as the growth and immunity promoters in it.

Dauda *et al.* (2018) worked on glycerol-based biofloc production and noticed its effects on the growth, physiological status and disease resistance of African catfish (*Clarias gariepinus*). Ten fish/replicates were challenged with the bacterial pathogen, *Aeromonas hydrophila*. After challenging the catfish to *A. hydrophila*, survival was significantly higher ($P < 0.05$) in the C/N 15 and 20 groups, which was accompanied with less histopathological liver damage compared to those in the control or C/N 10 treatment. Overall, the results indicate that in a glycerol-based

biofloc system, a C/N ratio of 15 led to the best balance of better water quality, nutritive value of *C. gariepinus* as well as their resistance to *A. hydrophila*.

1.1.3.3 Biofloc as additional food source

Formulated feed for freshwater prawn farming under semi-intensive conditions is expensive; presenting a significant portion of the farm's operating costs. Production and availability of natural food in earthen ponds may reduce the needs for formulated feed (Correia *et al.*, 2003). The biofloc technology increases the crude protein level and dry weight of the floc materials. Microbial protein of biofloc can replace fishmeal or other protein sources in fish and shrimp feeding due to its good nutritional value and appropriate amino-acid profile which make it suitable for feeding omnivorous cultured species (Hussain *et al.*, 2014).

In BFT, excess of nutrients in aquaculture systems are converted into microbial biomass, which can be consumed by the cultured animals as a food source. This technology, to a certain extent, has also the capacity to control pathogens in aquaculture. The benefits of BFT are also evident in the freshwater fish culture to include improved production through lower feed conversion rate, better nutrition, optimum water quality and health (Choo *et al.*, 2015).

Biofloc diets enhance shrimp growth, lower FCR, reduce the nitrate, nitrite-N levels and TAN in the experimental greenhouses, have a lower total feed cost in addition to better water quality and best shrimp economical production compared to conventional control diet. The biofloc treatments really succeed to reduce the cost of per kg shrimp production (Megahed, 2010).

Anand *et al.* (2013) observed the effects of C: N ratio and substrate integration on periphyton biomass, microbial dynamics and growth of *Penaeus monodon* juveniles. Addition of substrate and higher C:N ratio (CN20+S) significantly reduced ($p < 0.01$) inorganic nitrogen. Substrate addition significantly ($p < 0.05$) reduced the turbidity. Periphyton biomass and total heterotrophic bacterial load were significantly higher ($p < 0.05$) in CN20+S in comparison with CN10+S treatment. Provision of substrate and carbohydrate addition resulted in the highest body weight through provision of natural food in the form of periphytic algae and heterotrophic bacterial community. Treatment with substrates showed a higher survival rate by 7 % in comparison with without substrate treatments as it acted as shelter to shrimp during molting and there by reduced



cannibalism. Thus, the results demonstrated that high C: N ratio and substrate addition improved growth, reduced FCR and better water quality conditions.

1.1.3.4 Biofloc in aquaculture environment

Development of aquaculture industry is restricted due the undesirable environmental impact associated with the effluent discharge that is rich in inorganic nitrogenous compound and organic matters. This situation could be improved through the implementation of Bioflocs Technology (BFT). BFT can enhance the water quality in aquaculture system by introducing natural nutrient recycling characteristics with the induction of appropriate carbon/nitrogen (C/N) ratio. However, the natural condition of aquaculture system, which is rich in inorganic nitrogen, could not sustain BFT. Thus, additional carbon is required to obtain a suitable C/N ratio for effective formation of bioflocs complex. The highest removal of ammonia is achieved at 98.7% with the formation of 92.5 mL L⁻¹ of bioflocs concentration at an optimum C/N ratio of 15. The utilization of BFT can potentially provide a sustainable and low maintenance treatment of aquaculture system (Bakar *et al.*, 2015).

Biofloc technology (BFT) has beneficial effects in aquaculture management, including water quality, feeding and disease control. Application of BFT in aquaculture offers a solution to avoid the environmental impact of high nutrient discharges and to reduce the use of artificial feed (Choo *et al.*, 2015). The biofloc nursery system is a profitable alternative for locations where climatic and water restrictions do not allow traditional prawn cultivation and also contributes to sustainable use of water and improved nutritional quality of the prawns (Pérez-Fuentes *et al.*, 2013).

In 2016, Rajkumar, et al. noticed the effect of different biofloc system on water quality, biofloc composition and growth performance in *Litopenaeus vannamei*. They stated that wheat flour effectively enhances the biofloc production and contributes towards better water quality which results in higher production of shrimp. The experiment was conducted with three biofloc treatments fed with sugarcane molasses, tapioca flour, wheat flour and clean water as control without biofloc and allowed to stand for 30 days. The proximate and chemical composition of biofloc and proximate composition of the shrimp have significant difference between the biofloc treatments and control. All the biofloc are composed with tintinids, ciliates, copepods,

cyanobacteria and nematodes where nematodes are the most dominant group of organisms (Rajkumar, et al., 2016).

Turbidity values are superior quantifying parameters for quantitative determination of biofloc than total suspended solid in a biofloc-based system. Once a mature biofloc community is established, TAN and NO_2^- -N concentrations can be effectively controlled by either heterotrophic assimilation or autotrophic nitrification that helps maintain their concentrations at acceptable ranges for shrimp culture even at high stocking densities. The mixed type of biofloc dominated by both microalgae and autotrophic bacteria is more beneficial for shrimp performance in high density zero-exchange culture systems than systems dominated by heterotrophic bacteria, and also reduces production costs by reducing organic carbon input and oxygen use (Xu *et al.*, 2015).

Hutabarat et al. (2013) worked with different C: N and C: P ratio of media to see the effect on the content of poly hydroxyl-butyrate in biofloc inoculated with bacterium *Bacillus cereus*. Treatments were C: N ratio of 15, 20, 25 and C: P ratio of 75, 100, and 125. The result showed that C: N ratio and C:P ratio of media and their interaction affect the content of poly hydroxy-butyrate in biofloc.

In microscopic view, there have abundances and high varieties of phytoplankton, zooplankton, protozoa, nematodes and algae species which aggregates together in the flocs accumulation. All of these microscopic organisms implement the symbiotic process together for food supply, become the algae grazer, act as natural water stabilizer in regulating the nutrients and serve as decomposer for dead organic matter in the water environment. Heterotrophic bacteria from *Pseudomonas* and *Aeromonas* family consume the organic matter loaded at the bottom of culture area and converts items through chemical process as useful protein food to be consumed back by the shrimp. Overall, the biofloc organisms really contributes as natural bioremediation agents in zero water exchange culture system of Pacific white shrimp, *Penaeus vannamei* to ensure the water quality in the optimal condition (Manan *et al.*, 2016).

Mabote et al. conducted a experiment to evaluate the performance of *Penaeus monodon* postlarvae (PL) reared during the nursery phase in a BFT limited water exchange system. The BFT treatment show higher water quality parameters with the exception of dissolved oxygen.

The mean final weight, growth rate and biomass in the BFT treatment is higher than the clean water treatment ($P < 0.05$). FCR is lower ($P < 0.05$) in the BFT treatment than the clean water treatment. Actually, a BFT system improves the growth performance of *P. monodon* and maintains the water quality within a range which is considered optimal for the species (Mabote, et al., 2017).

Shrimp (*Litopenaeus stylirostris*) broodstock reared with Biofloc technology (BFT) compared to clear water exhibit a higher health status with a better final survival rate during the reproduction period and higher glutathione level (GSH) and total antioxidant status (TAS), reduced oxidized/reduced glutathione ratio and a higher spawning rate and frequency as well as higher gonado-somatic index and number of spawned eggs. Finally, larvae from broodstock from BFT exhibit higher survival rates at the Zoe 2 and Post Larvae 1 stages when compared with those from females from clean water treatment. The improved reproductive performance of the broodstock and higher larvae survival rate resulting from BFT treatment may be linked to the dietary supplement obtained by the shrimp from natural productivity during BFT rearing. Indeed, biofloc particulates represent a potential source of dietary glutathione and a significant source of lipids, particularly essential phospholipids and n-3 highly unsaturated fatty acids (HUFA) for shrimps. Thus, broodstock from BFT treatment accumulated phospholipids, n-3 HUFA and arachidonic acid, which are necessary for vitellogenesis, embryogenesis and pre-feeding larval development (Emilie *et al.*, 2016).

In 2012, Ray et al., explored the effects of biofloc concentration reduction and a fish-free diet on several microbial community characteristics in super intensive Shrimp, *Litopenaeus vannamei*, culture systems. Photosynthetically active radiation (PAR) extinction coefficients, photosynthetic oxygen production, chlorophyll-a (chl-a) concentrations, pheophytin-a (pheo-a) concentrations, and the sum of odd and branched chain fatty acid concentrations as a bacterial abundance indicator (BAI) were measured. Biofloc reduction significantly ($P \leq 0.003$) decreased PAR extinction coefficients, chl-a concentration, pheo-a concentration, and BAI concentration, while significantly increasing photosynthetic oxygen production. Diet did not significantly affect ($P > 0.05$) any of these measured parameters. The observed changes in microbial community characteristics corresponded with, and may help to explain; significantly improved shrimp feed

conversion ratios, and growth rate, final weight, and biomass yield in the tanks with biofloc reduction.

In 2013, an experiment was conducted by Magondu *et al.* to observe the effects of C/N ratios and stocking density on water quality, sediment quality and growth of *Labeo victorianus*. Increasing C/N ratio from 10 to 20 reduces the total ammonia nitrogen (TAN), nitrite–nitrogen ($\text{NO}_2\text{-N}$) and nitrate–nitrogen ($\text{NO}_3\text{-N}$) in the water column and total nitrogen in the sediment. It also raises sediment pH, organic matter and total phosphorus. The lowest protein efficiency ratio (PER), specific growth rate (SGR) and the highest food conversion ratio for the feed are gained with a C/N ratio of 10. Based on highest growth, survival, production and net benefits, C/N ratio of 20 and a stocking density of 25 fish m^{-2} are optimal. Therefore, carbohydrate addition in *L. victorianus* culture is a promising option for sustainable aquaculture (Magondu *et al.*, 2013).

Integrated Multi-Trophic Aquaculture (IMTA) is an ecological and economic farming strategy that minimizes waste from culture systems, reduces the risk of diseases and provides additional income source. The addition of starch into the IMTA system of shrimp, spotted scat and water spinach improved productivity, profitability and water quality. The combination of the IMTA model and biofloc technology have a synergistic effect on overall FCR and economic return, and was more effective at improving shrimp survival, production and reducing nitrite-N and TAN than use of the IMTA model alone (Liu *et al.*, 2014).

In 2017, Miao *et al.* performed an experiment to evaluate the effects of biofloc technology in combination with probiotics based on a rearing system on the growth and immunity of giant freshwater prawn (*Macrobrachium rosenbergii*). The study suggested that rearing prawns using in situ biofloc technology combined with the addition of probiotics can enhance nonspecific immune responses and disease resistance under zero-water-exchange systems, sustain the homeostasis of intestinal microbiota and improve the absorption area of the intestine by increasing intestinal HF, which ensures the sustainability of *M. rosenbergii* culture. Giant freshwater prawn reared using in situ biofloc technology in combination with probiotics could enhance non-specific immune responses, disease resistance under zero water exchange system, sustain the intestinal microbiota homeostasis and improve the absorption area of the intestine by increasing intestinal fold height, which ensured the sustainability of *M. rosenbergii* culture.

Another experiment was conducted by Effendy et al. in 2016 to assess the growth and production of Indian white prawn, *Fenneropenaeus indicus* in semi intensive and semi biofloc culture technique. Phytoplankton population and total haemocyte count were found to be increased and pathogenic bacteria population decreased in treatment ponds. Strong linear relationship was found between growth and biofloc content in treatment ponds. Shrimp grown in biofloc pond showed better colour and taste compared to control. Semi biofloc technique can be considered as an ideal culture method for bio secure production of white shrimp in semi-arid lands.

1.1.4 Literature gap

From the above study, it was assumed that there had no remarkable findings on bacterial count from the biofloc in prawn culture system. So, this present study was conducted by following the methodology given in the next chapter.



Chapter Two

MATERIALS AND METHODS



2 Materials and methods

2.1 Study area and period

The trials for biofloc development were carried out in the fiber glass tank in water chemistry laboratory of Fisheries and Marine Resource Technology (FMRT) Discipline, Khulna University and in the Molecular Biology Laboratory of same Discipline from 02, August to 15, August, 2017. Biofloc volume was determined at the end of the tank trials and samples were for the bacterial composition of the flocs was determined for next one month. For the determination of the bacterial composition, the freshwater prawn *Macrobrachium rosenbergii* were cultured with BFT treatment in experimental tank and then the samples were taken in the Biochemistry and Molecular Genetics Laboratory of FMRT Discipline, Khulna University for culture and enumeration of bacterial species.

2.2 Experimental Design

The experiment was conducted in the fiberglass tank for close observation of the biofloc technology. The experimental tanks was treated with the C:N=10:1 (denoted as T₁), C:N=15:1 (denoted as T₂) and C:N=20:1 (denoted as T₃) to develop the biofloc. In addition, a control group was also maintained without any carbon addition. The heterotropic bacteria were developed in the flocs, which was collected and determined from each of the experimental groups. Afterwards, macro and microbiological composition of the flocs was determined by laboratory analysis. Macroscopic organisms were determined by using light microscope and the potential bacterial composition was determined by microbiological plate assay. The floc samples were examined to observe the load of *Enterococcus* spp., *Clostridium* spp., *Lactobacillus* spp. and *Salmonella* spp. The whole study was conducted by maintaining the following experimental design (Figure 2.1):

Figure 2.1 Layout of the experiment for *M. rosenbergii* culture using biofloc technology at differernt C:N ratios.

Treatments	Description of Treatment	Replications
C	Control	3
T ₁	Treated with Biofloc technology (C:N= 10:1)	3

T ₂	Treated with Biofloc technology (C:N= 15:1)	3
T ₃	Treated with Biofloc technology (C:N= 20:1)	3

2.3 Development of Biofloc Technology

Twelve tanks were set randomly in the water chemistry laboratory for developing bioflocs in prawn culture system. Before stocking the prawn juveniles were weighed and feed was given at 5% of their body weight. From the amount of feed, the proper amount of molasses was determined to maintain proper C/N ratio (10, 15 and 20) in different treatments. After that, specific amount of feed and molasses were provided to the experimental tanks twice a day to adjust the feed nitrogen for targeted microbial growth. Besides, continuous aeration was provided in all the tanks for homogenous oxygen and nutrients distribution for flocs.

2.4 Laboratory Requirements for microbial assay

2.4.1 Laboratory Equipment

Microscope, autoclave machine, centrifuge machine, electric balance, spirit lamp and burning spirit, petri dish, biological dissection box, solution mixer, bacteria isolating loops, refrigerator, bio-safety cabinet, micro-pipette, tips and tips box (Swanson *et al.*, 2001) were required for conducting the study.

2.4.2 Reagents and chemicals

Peptone water, Absolute alcohol, Ethyl alcohol (75%), distilled water and detergent powder were needed during the study periods (Gavin & Cummings, 1989).

2.4.3 Types of media needed during culture period

Plate counting agar was used for standard plate count. For culture and normal counting of *Salmonella* spp., Broth Medium I (Tetrathionate Bile Brilliant Green Broth) was required. This media was specific or selective for harmful *Salmonella* spp. For culture and normal counting of *E. faecalis*, SF Broth Medium was used. *Lactobacillus* spp. bacteria were cultured in *Lactobacillus* MRS Agar medium. For culture and normal counting of *Clostridium butyricum*, selective Reinforced Clostridial Agar (M154) was used.

2.5 Sample collection

Samples of floc were collected for three times from each of the experimental tank for each and every enumeration at every three days interval. So, nine samples were collected under each treatment. As a result thirty six samples were collected from twelve experimental tanks under four treatment groups. Then collected sample was carried immediately to the Molecular Biology Laboratory of FMRT Discipline, Khulna University. All the samples were instantly used for microbial analysis and also properly labeled and stored in -20°C temperature for further analysis.

2.6 Laboratory work

2.6.1 Floc Determination

Total volume of floc per liter water was measured at first by using specific cone. Then the proportion or percentage of different types of flocs such as protozoa, plankton, copepods, etc. in the collected sample was determined at first by observing them under a light microscope.

2.6.2 Sterilization of equipment

Test tubes, Petri dishes, conical flask, plastic tips and other glassware were sterilized by autoclaving at a temperature of 121°C and a pressure of 15 pound per square inch for 15 minutes. Petri dishes, tips box was subjected to dry heat sterilization at 155°C for 1 hour in electric oven (Swanson *et al.*, 2001).

2.6.3 Sample preparation and dilution

Accurate amount (1 ml) of pooled sample was measured by pipette. Then the sample was taken into a sterile test tube containing 9 ml 0.1% sterile peptone water for vortex mixing. The mixture was homogenised for 2 min. This serial dilution was continued up to 10^{-5} . Samples from each of the experimental tank were taken and prepared in the same way. The prepared samples of the six tanks were kept in separate test tube.

2.6.4 Total bacteria count

At first the samples was cultured in the standard plate count agar media. Then the colonies of the total heterotrophic bacteria were counted by using colony counting equipment.

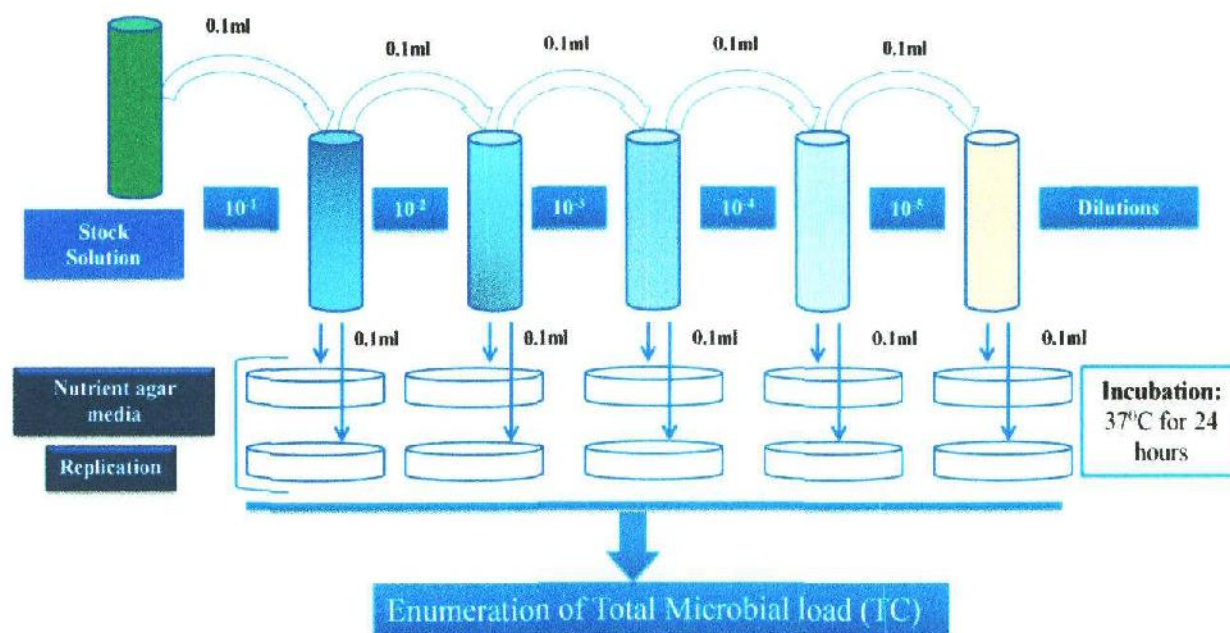


Figure 2.1 Enumeration of total bacteria loads from collected biofloc samples.

2.6.5 Determination of *Enterococcus* spp.

2.6.5.1 Composition of culture media

Enterococcus spp. bacteria were cultured in the SF Broth media. SF Broth was produced by Hi Media Laboratories according to the formulation developed by Hajna and Perry (1943). The media composition has been given in the section of appendix-1.

2.6.5.2 Preparation of culture media

36.032 g media (SF Broth) was mixed in 1000 ml of distilled/ deionized water. Heat was provided to dissolve the medium completely. Then it was dispensed in tubes and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. The Final pH of the media was adjusted to 6.9 ± 0.3 at 25°C before autoclaving. The media was stored below 30°C in tightly closed container and the prepared medium was at 2 - 8°C.

2.6.5.3 Culture of *Enterococcus* spp.

Then the bacteria were cultured in the prepared culture media (SF broth). The bacteria were cultured in five-fold dilutions and each dilution had three tubes. So, fifteen test tubes were needed for one stock solution. Twelve stock solutions were prepared from the twelve experimental tanks and used for bacterial analysis. $(12 \times 15) = 180$ test tubes were needed for the culture of bacteria of twelve tanks. The test tubes were incubated at specific temperature (45.5°C) for bacterial growth.

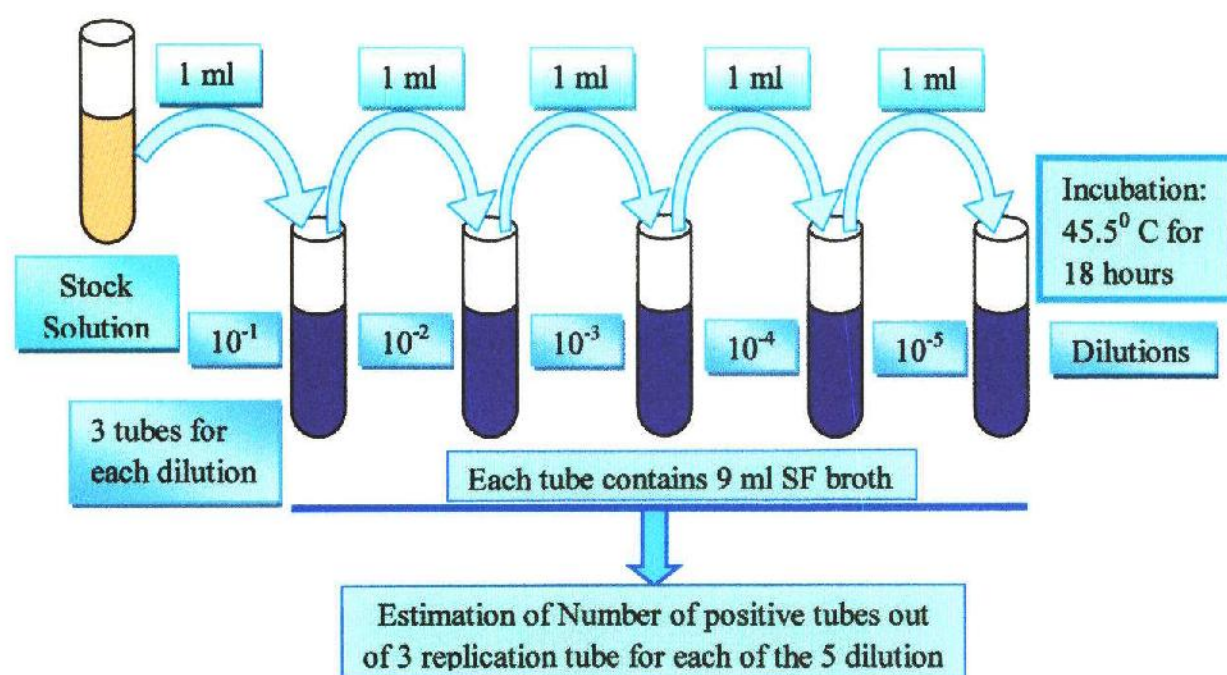


Figure 2.2 Flow chart of culture techniques of *Enterococcus* spp. in SF broth.

2.6.5.4 Cultural Response *Enterococcus* spp.

Cultural characteristics were observed after incubation at $45\text{--}46^\circ\text{C}$ for 18–48 hours. The color of the tubes were changed from purple to yellow giving the positive result in which the growth *Enterococcus* spp. was luxuriant. Some tubes remained unchanged (purple color) which gave the negative result due to lack of growth. As the SF broth media is selective for Enterococci and the presence of *Enterococcus* spp. was identified by the color change (from purple to yellow) at the time of culture, so any further confirmation test was not necessary.



Figure 2.3 Cultural response of *Enterococcus* spp. in SF broth after incubation.

2.6.5.5 Data collection of *Enterococcus* spp.

In the tube culture method, the number of bacteria cannot be found directly. Only the number of positive and negative tube for each dilution were found and recorded. Then an established Most Probable Number (MPN) chart was used to estimate the number of bacterial cell by using the number of positive tube for each dilution. The MPN chart was prepared by J.L. Oblinger and J.A. Koburger in 1975 on the basis of poisson counting statistics regarding the number of positive tubes out of all the cultured tubes. The bacterial culture was done in a five-fold dilution each of with three replication tubes. So, three tubes MPN chart was used for the estimation of the load of *Enterococcus* spp. Then the Most Probable Number (MPN) bacterial data was recorded, accumulated, grouped and interpreted according to the objectives as well as parameters.



Figure 2.4 Estimation of the number of positive tubes out of all the tubes for each of the dilution separately

2.6.6 Determination of *Clostridium* spp.

2.6.6.1 Composition of culture media

Clostridium spp. bacteria were cultured in the Reinforced Clostridial Agar (M154) media. The media composition has been given in the section of appendix-1.

2.6.6.2 Preparation of culture media

51.0 gm of the media was dissolved in 1 L of distilled water, followed by heat sterilization by autoclaving at 15 lbs pressure (121°C) for 15 minutes. By pour plating the bacteria was inoculated into the media and incubated at anaerobically at 37 °C for 24 hours (Chaturvedi and Shukla, 2015).

2.6.6.3 Culture of *Clostridium* spp.

Clostridium spp. was cultured by Pour plating method. The appropriate dilutions were selected and for every dilution 1.0 ml aliquot were transferred into sterile petri dishes. Approximately 20.0 ml portion of Reinforced Clostridial agar was poured into each of these sterile petri dishes. The plates were then rotated 5 times clockwise, 5 times anticlockwise, 5 times back and forth. Care was taken not to splash agar on the lid of the dish. Plates were left to solidify the plates and

were inverted. Then the Petri dishes with solidified agar were incubated at 37°C for 24 h in the incubator.

2.6.7 Determination of *Lactobacillus* spp.

2.6.7.1 Composition of culture media

Clostridium spp. bacteria were cultured in the Lactobacillus MRS Agar medium. The media composition has been given in the section of appendix-1.

2.6.7.2 Preparation of culture media

About 67.15 g of the media was dissolved in 1 L of distilled water, followed by heat sterilization by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

2.6.7.3 Culture of *Lactobacillus* spp.

Lactobacillus spp. was cultured by pour plating method same as the *Clostridium* spp. culture and incubated for 18-48 hours at 35-37 °C.

2.6.8 Determination of *Salmonella* spp.

Salmonella spp. bacteria was cultured in the Broth Medium I (Tetrathionate Bile Brilliant Green Broth media). The composition of the media has been given in the section of appendix-1.

63.07 gm of the media was dissolved in 1 L of distilled water, followed by heat sterilization by autoclaving at 15 lbs pressure (121°C) for 15 minutes. By pour plating method the bacteria was inoculated into the media and incubated at anaerobically at 41-43°C for 18-24 hours.

2.6.9 Counting of colonies

After the specified period of incubation the bacterial colonies of each Petri dish were counted, using the colony counting equipment. Plates with 10-300 colonies on the surface were only counted. To calculate the number of bacteria per mL of diluted sample following equation was used (Herigstad *et al.*, 2001):

$$\frac{\text{Number of CFU}}{\text{Volume plated (mL)} \times \text{total dilution used}} \longrightarrow \frac{\text{Number of CFU}}{\text{mL}}$$

2.7 Data analysis

The data was recorded during the experiment and collected data were stored, explored and analyzed using Microsoft Excel, SPSS and Microsoft Word program to present the results and discussion. The normality test of the data was done in SPSS at first. After verifying the normality of flocs volume and log transformed bacterial data, one-way analysis of variance (one-way ANOVA) was performed to check the significant differences among the treatments. After detecting it ($P < 0.05$), the Tukey test of means comparison was used in SPSS to compare the data of control and treated tanks.

2.8 Biochemical tests of bacterial composition

For the confirmation of specific bacterial species like *Enterococcus* spp., *Clostridium* spp., *Lactobacillus* spp., the following biochemical tests was conducted:

1. D- Sorbitol test
2. D- Xylose test
3. L- Arabinose test
4. Sulphur Indole Motility (SIM) test
5. Triple Sugar Iron (TSI) agar test
6. Indole test
7. Methyl Red test
8. Voges Proskauer test
9. Catalase test
10. Oxidase Test
11. Starch hydrolysis test



Chapter Three

RESULTS



3 RESULTS

3.1 Floc Determination

At every three days interval, the volume of floc per liter was measured for three times from each of the tank of treatment groups. C/N ratio 15 and 20 (T2 and T3) showed higher floc development compared to C and T1 ($P < 0.05$). The lowest volume of floc was determined in C group, 5.22 ± 0.62 ml/L, followed by $7.22 \pm .46$ ml/L, 12.22 ± 1.08 ml/L and 15.33 ± 1.37 ml/L T1, T2, and T3, respectively (Figure 4.1).

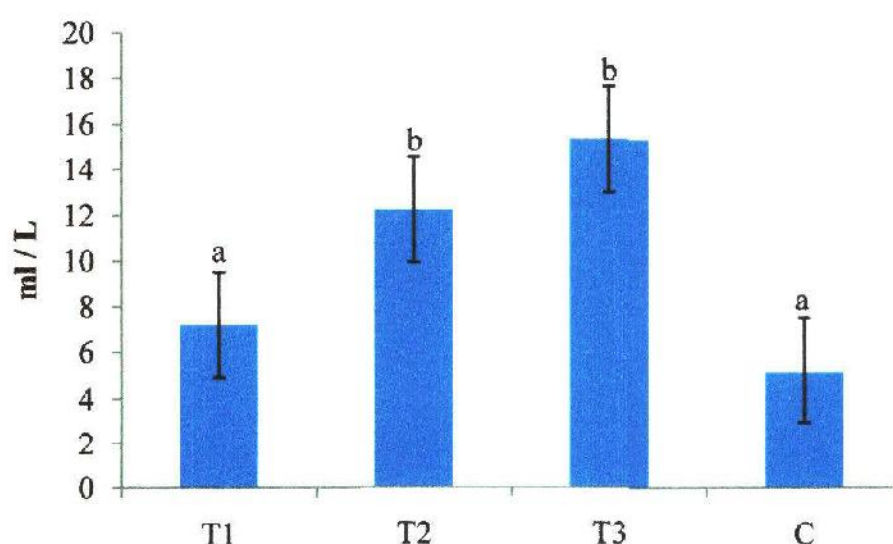


Figure 3.1 Volume of flocs at different C/N ratios in biofloc based prawn culture system. The error bars represent the standard deviations of nine replicates. Different letters indicate significant difference among the treatments (one-way ANOVA, $P < 0.05$).

3.2 Total bacterial count

The mean number of total heterotrophic bacteria (THB) in control group was $(1.56 \pm 0.07) \times 10^7$ CFU/ ml which was lower than T2 $((2.30 \pm 0.21) \times 10^8$ CFU/ ml) and T3 $((3.90 \pm 1.33) \times 10^8$ CFU/ ml) ($P < 0.05$) (Figure 3.2). The treatment with C/N, 10 also showed significantly lower THB $((1.55 \pm 0.28) \times 10^7$ CFU/ ml) compared to T2 and T3 ($P < 0.05$) (Figure 3.2).

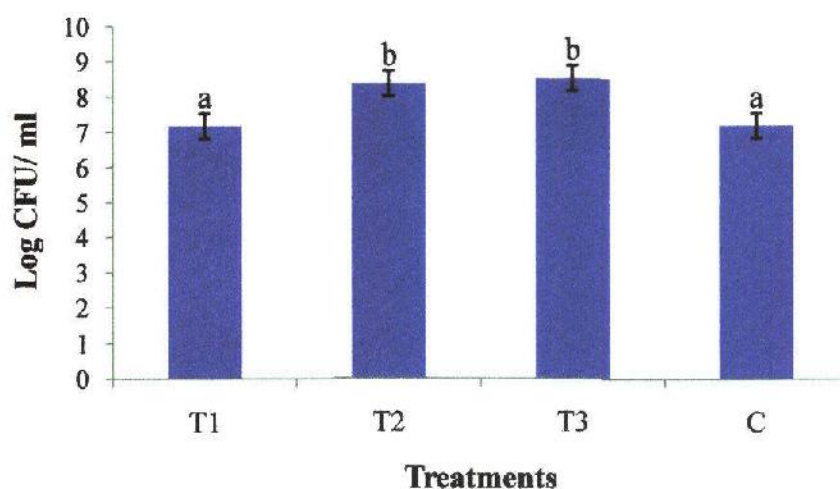


Figure 3.2 Total heterotrophic bacterial count (Log CFU/mL) in the flocs at different C/N ratios in biofloc based prawn culture system. The error bars represent the standard deviations of nine replicates. Different letters indicate significant difference among the treatments (one-way ANOVA, $P < 0.05$).

3.3 *Enterococcus* spp. count

The mean number of *Enterococcus* spp. was the highest in T2 (2.74 ± 0.30) $\times 10^3$ MPN/ ml compared to other treatments ($P < 0.05$). The load of this beneficial bacteria was found higher also in T1 ($(1.29 \pm 0.21) \times 10^3$ MPN/ ml) and C ($(1.19 \pm 0.09) \times 10^3$ MPN/ ml) compared to T3 ($(4.18 \pm 0.88) \times 10^2$ MPN/ ml) ($P < 0.05$) (Figure 3.3).

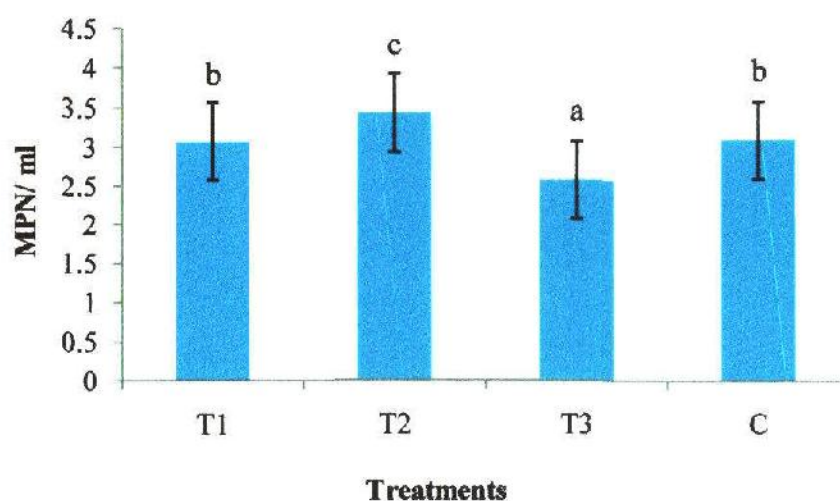


Figure 3.3 *Enterococcus* spp. count (MPN /mL) in the flocs at different C/N ratios in biofloc based prawn culture system. The error bars represent the standard deviations of nine replicates. Different letters indicate significant difference among the treatments (one-way ANOVA, $P < 0.05$).

3.4 *Clostridium* spp. count

The highest mean number of *Clostridium* spp. was found in T1 ($(8.64 \pm 1.74) \times 10^5$ CFU/ ml), followed by T2 ($(6.89 \pm 2.16) \times 10^5$ CFU/ ml), T3 ($(4.74 \pm 1.75) \times 10^5$ CFU/ ml) and Control group ($(3.59 \pm 0.87) \times 10^5$ CFU/ ml) respectively. The treatments did not show significant difference among them in respect of *Clostridium* spp. load in the flocs (Figure 3.4) ($P < 0.05$).

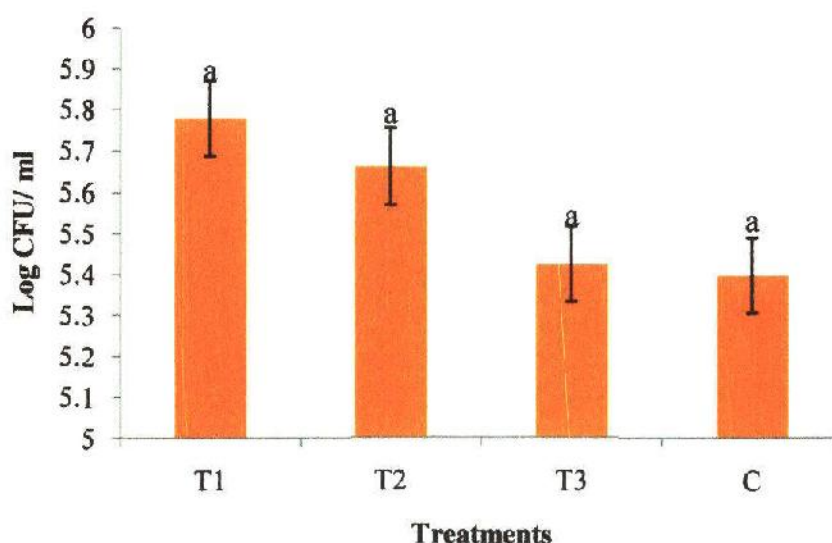


Figure 3.4 *Clostridium* spp. count (LogCFU/mL) in the flocs at different C/N ratios in biofloc based prawn culture system. The error bars represent the standard deviations of nine replicates. Different letters indicate significant difference among the treatments (one-way ANOVA, $P < 0.05$).

3.5 *Lactobacillus* spp. count

The mean number of *Lactobacillus* spp. was found to be higher in T1 compared to T3 and C ($P < 0.05$). T1 had the highest number of *Lactobacillus* spp. count ($(1.15 \pm 0.12) \times 10^6$ CFU/ ml) among the treatments, followed by T2 ($(9.65 \pm 2.48) \times 10^5$ CFU/ ml), C ($(3.02 \pm 0.95) \times 10^5$ CFU/ ml) and T3 ($(2.53 \pm 0.25) \times 10^5$ CFU/ ml) (Figure 3.5).

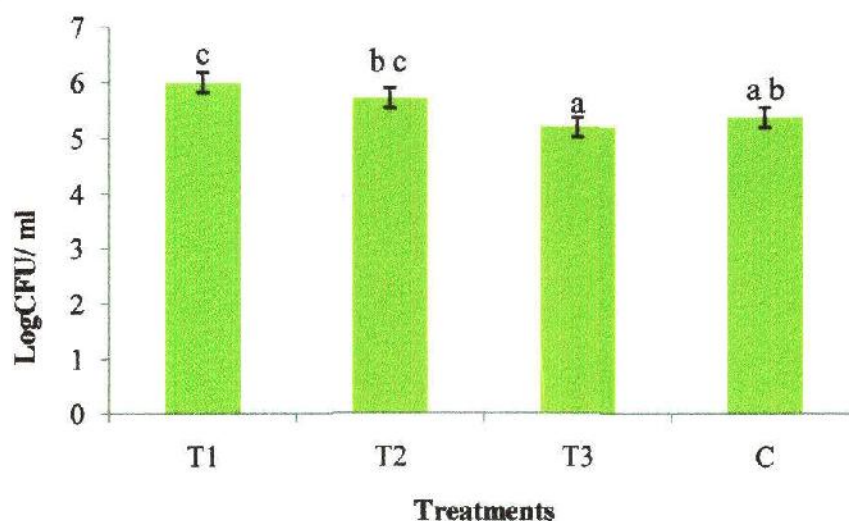


Figure 3.5 *Lactobacillus* spp. count (LogCFU/mL) in the flocs at different C/N ratios in biofloc based prawn culture system. The error bars represent the standard deviations of nine replicates. Different letters indicate significant difference among the treatments (one-way ANOVA, $P < 0.05$)

3.6 *Salmonella* spp. count

No colony forming units of *Salmonella* spp. was found in any of the treatment in this study.

3.7 Biochemical tests

Result of Biochemical tests found for the confirmation of bacterial species has been mentioned in the following Table.

Table 3.1 Results of biochemical tests for indication of specific group of bacteria.

Name of the Tests	Results for <i>Enterococcus</i> spp.	Results for <i>Clostridium</i> spp.	Results for <i>Lactobacillus</i> spp.
D- Sorbitol test	Positive (+)	Positive (+)	Positive (+)
D- Xylose test	Negative (-)	Negative (-)	Positive (+)
L- Arabinose test	Positive (+)	Negative (-)	Negative (-)
Sulphur Indole Motility (SIM) test	Negative (-)	Negative (-)	Negative (-)

Triple Sugar Iron (TSI) agar test	Positive (+)	Positive (+)	Positive (+)
Indole test	Negative (-)	Negative (-)	Positive (+)
Methyl Red test	Negative (-)	Negative (-)	Negative (-)
Voges Proskauer test	Negative (-)	Negative (-)	Negative (-)
Catalase test	Negative (-)	Negative (-)	Negative (-)
Oxidase Test	Negative (-)	Negative (-)	Negative (-)
Starch hydrolysis test	Positive (+)	Positive (+)	Negative (-)



Chapter Four

DISCUSSIONS



4 Discussion

In this study, the C/N ratio was necessarily correlated with the floc volume. The volume was increased with the increasing rate of C/N ratio. But the density of floc was lower in C/N 20. The best quality floc and the highest volume were found in the C/N 15. This also appears to be supported by the bioflocs appearing more closely aggregated and being less dense in the C/N 10 and 20 groups compared to the biofloc particles in the C/N 15 treatment, which is consistent with filamentous bacteria formation (Xu et al., 2016).

The volume was also between 7 to 15 ml/ L in the treatments that have similarities with the statement of Avnimelech (1999). Proliferation of bacterial colonies and microorganisms generates an increase in the biofloc biomass, it increase must have a density between 10 and 15 mL so the system can keep functioning properly between 7 to 15 ml/ L in the treatments that have similarities with the statement of Avnimelech (1999).

The addition of carbohydrate to the water column increased the C/N ratio and resulted in a significant increase in the total heterotrophic bacterial (THB) count. Similar results were found by Burford et al. (2003) and Hari et al. (2004). Mean THB count of the flocs varied from 41.5×10^7 to 53.9×10^7 CFU/ml revealed the positive effect of carbohydrate addition on the bacterial population of the pond sediment. The number of bacteria showed a significant increase from the initial sampling date to the second and then stabilized for the entire culture period (Hari et al., 2004).

Generating a nitrogen cycle in a closed water system by stimulating the microbial growth is the basic principle of biofloc technology (Ekasari et al., 2015). Molasses, which is a by-product of sugar production that contained 49 – 50% carbon (Paturau, 1982) was used in the experiment. The higher bacterial population in the carbohydrate added treatments revealed that the molasses is a good source of organic carbon as it was well utilized by the heterotrophic bacterial population. Anand et al. (2014) used wheat flour as a carbon source to stimulate microbial growth and found the main microbes to be *Vibrio*, *Lactobacillus*, *Bacillus* and fungi. Ferreira et al. (2015) isolated *Bacillus* from a highly enclosed culture system of *L. vannamei*.

In the present study, the highest load of *Enterococcus* spp. was found in T2 and it had significant difference with the control and other C/N treated systems. *Enterococcus* spp. load was lowest in T3 at the C/N, 20 might be due to over organic load which might cause development of toxic gases, lowering the pH of the system, thereby creating unfavourable condition for growth of different bacterial population. Similarly, load of *Lactobacillus* spp. was also lower in T3 compared to other treatments, which was found to be highest in T1 and T2. These findings have similarities with the study of Pacheco-Vega et al. (2018). They reported that the total count of viable heterotrophic bacteria of the genus *Bacillus* spp. in the water of shrimp cultures demonstrated that during the culture that *Bacillus* spp. presented the greatest bacterial load in both treatments. Miao et al. (2017) also found that in the biofloc tanks, *Bacillus subtilis* and *Lactococcus* could utilize the nitrogenous waste and accumulated carbon as a nutrient source to preserve shrimp growth in the water. The growth of *Bacillus subtilis* and *Lactococcus* was restricted in the control tanks because of an inadequate carbon source. Therefore, biofloc technology played an important role in this study in maintaining a relatively high proportion of *Bacillus subtilis* and *Lactococcus* in the biofloc rearing water. *Bacillus subtilis* and *Lactobacillus* can successfully colonize the intestine and affect the intestinal microbial community (Miao et al. 2017). The microbial community in the host intestine has been reported to play a key role in improving the immunity of the host by forming a physical barrier to provide a defense against pathogens or by occupying an ecological niche (Heijtz et al., 2011; Maslowski and Mackay, 2011).

However, although C/N, 10 and 15 revealed higher count of *Clostridium* spp., but the load was not significant with either T3 or Control group. This phenomenon might be due to the short culture period for the proper development of these bacteria. It was similar to the study of Ferreira et al. (2015) that isolated *Bacillus* spp. from the super-intensive marine shrimp system with biofloc, added cells in culture tanks, and did not observe significant differences in heterotrophic bacteria count.

Finally, the abundance of *Salmonella* spp. was nil. It assumes that the environment of the culture system was good and hygienic and the beneficial microbes might inhibit the growth of the pathogenic agent. Like as, the association of flocs acts as a bioregulator of water quality and as an inhibitory agent to prevent the growth of *Vibrio* spp. during the cultivation of shrimp in the

biofloc system (Pacheco-Vega et al., 2018). Moreover, the addition of lactic acid bacteria in shrimp diets has been suggested to decrease the adherence and colonization of pathogenic bacteria and to improve fish health (Ringø et al., 2010). So, the *Lactobacillus* spp. may be the cause of inhibiting the *Salmonella* spp. Many other studies have also found that biofloc technology directly led to significantly different microbial composition in the rearing water (Anand et al., 2013; Tapia-Paniagua et al., 2014; Ferreira et al., 2015).

Chapter Six

REFERENCES

6 References

- Abbaszade A, Yavari V, Hosseini S, Nafisi Bahabadi M. (2018). Effects of molasses and spoilage date palm extract on bacterial load of water, growth performance and intestine of Pacific white shrimp (*Litopenaeus vannamei*) in the biofloc technology. Iranian fisheries science research institute. 26 (5) :33-41
- Ajmal Hussan, T. G. Choudhury, Vinay T. N., S. K. Gupta, (2016). Common problems in aquaculture and their preventive measures. Aquaculture Times, 2(5).
- Anand, P. S., Kohli, M. P. S., Kumar, S., Sundaray, J. K., Roy, S. D., Venkateshwarlu, G., ... & Pailan, G. H. (2014). Effect of dietary supplementation of biofloc on growth performance and digestive enzyme activities in *Penaeus monodon*. *Aquaculture*, 418, 108-115.
- Anand, P. S., Kumar, S., Panigrahi, A., Ghoshal, T. K., Dayal, J. S., Biswas, G., ... & Pillai, S. M. (2013). Effects of C: N ratio and substrate integration on periphyton biomass, microbial dynamics and growth of *Penaeus monodon* juveniles. *Aquaculture International*, 21(2), 511-524.
- Asaduzzaman, M., Rahman, M. M., Azim, M. E., Islam, M. A., Wahab, M. A., Verdegem, M. C. J., & Verreth, J. A. J. (2010). Effects of C/N ratio and substrate addition on natural food communities in freshwater prawn monoculture ponds. *Aquaculture*, 306(1-4), 127-136.
- Austin, B., Austin, D. A., Austin, B., & Austin, D. A. (2012). *Bacterial fish pathogens* (p. 652). Heidelberg, Germany: Springer.
- Avnimelech Y (2007). Feeding with microbial flocs by tilapia in minimal discharge bioflocs technology ponds. *Aquaculture* 264:140–147.
- Avnimelech Y, Kochva M, Diab S (1994) Development of controlled intensive aquaculture systems with a limited water exchange and adjusted carbon to nitrogen ratio. *Bamidgeh* 46:119–131.
- Avnimelech Y. 1999a. Biofloc technology- A practical guide book. The world aquaculture society. Baton Rouge, United States.

- Avnimelech, Y. (1999). Carbon/nitrogen ratio as a control element in aquaculture systems. *Aquaculture*, 176(3-4), 227-235.
- Avnimelech, Y. (2007). Feeding with microbial flocs by tilapia in minimal discharge bio-flocs technology ponds. *Aquaculture*, 264(1-4), 140-147.
- Avnimelech, Y. (2009). Biofloc technology. *A practical guide book. The World Aquaculture Society, Baton Rouge*, 182.
- Avnimelech, Y., & Kochba, M. (2009). Evaluation of nitrogen uptake and excretion by tilapia in bio floc tanks, using ^{15}N tracing. *Aquaculture*, 287(1-2), 163-168.
- Azim, M. E., & Little, D. C. (2008). The biofloc technology (BFT) in indoor tanks: water quality, biofloc composition, and growth and welfare of Nile tilapia (*Oreochromis niloticus*). *Aquaculture*, 283(1-4), 29-35.
- Bakar, N. S. A., Nasir, N. M., Lananan, F., Hamid, S. H. A., Lam, S. S., & Jusoh, A. (2015). Optimization of C/N ratios for nutrient removal in aquaculture system culturing African catfish, (*Clarias gariepinus*) utilizing Bioflocs Technology. *International Biodeterioration & Biodegradation*, 102, 100-106.
- Bryan, F. L. (1977). Diseases transmitted by foods contaminated by wastewater. *Journal of food protection*, 40(1), 45-56.
- Burford MA, Thompson PJ, McIntosh RP, Bauman RH, Pearson DC (2004) The contribution of flocculated material to shrimp (*Litopenaeus vannamei*) nutrition in a high intensity, zero-exchange system. *Aquaculture* 232:525-537.
- Burford, M. A., Thompson, P. J., McIntosh, R. P., Bauman, R. H., & Pearson, D. C. (2003). Nutrient and microbial dynamics in high-intensity, zero-exchange shrimp ponds in Belize. *Aquaculture*, 219(1-4), 393-411.
- Cabello, F. C. (2006). Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. *Environmental microbiology*, 8(7), 1137-1144.

- Cadiz, R. E., Traifalgar, R. F. M., Sanares, R. C., Andrino-Felarca, K. G. S., & Corre Jr, V. L. (2016). Comparative efficacies of tilapia green water and biofloc technology (BFT) in suppressing population growth of green Vibrios and *Vibrio parahaemolyticus* in the intensive tank culture of *Penaeus vannamei*. *Aquaculture, Aquarium, Conservation & Legislation-International Journal of the Bioflux Society (AACL Bioflux)*, 9(2).
- Camargo, J. A., & Alonso, Á. (2006). Ecological and toxicological effects of inorganic nitrogen pollution in aquatic ecosystems: a global assessment. *Environment international*, 32(6), 831-849.
- Cardona, E., Lorgeoux, B., Chim, L., Goguenheim, J., Le Delliou, H., & Cahu, C. (2016). Biofloc contribution to antioxidant defence status, lipid nutrition and reproductive performance of broodstock of the shrimp *Litopenaeus stylirostris*: Consequences for the quality of eggs and larvae. *Aquaculture*, 452, 252-262.
- Carss, D. N. (1990). Concentrations of wild and escaped fishes immediately adjacent to fish farm cages. *Aquaculture*, 90(1), 29-40.
- Choo, H. X., & Caipang, C. M. A. (2015). Biofloc technology (BFT) and its application towards improved production in freshwater tilapia culture. *Aquaculture, Aquarium, Conservation & Legislation-International Journal of the Bioflux Society (AACL Bioflux)*, 8(3).
- Chu, C. P., & Lee, D. J. (2004). Multiscale structures of biological flocs. *Chemical Engineering Science*, 59(8-9), 1875-1883.
- Correia, E. S., Pereira, J. A., Silva, A. P., Horowitz, A., & Horowitz, S. (2003). Growout of freshwater prawn *Macrobrachium rosenbergii* in fertilized ponds with reduced levels of formulated feed. *Journal of the World Aquaculture Society*, 34(2), 184-191.
- Crab R, Kochva M, Verstraete W, Avnimelech Y (2009) Bio-flocs technology application in over-wintering of tilapia. *Aquac Eng* 40:105-112.
- Crab, R., Defoirdt, T., Bossier, P., & Verstraete, W. (2012). Biofloc technology in aquaculture: beneficial effects and future challenges. *Aquaculture*, 356, 351-356.

- Crab, R., Lambert, A., Defoirdt, T., Bossier, P., & Verstraete, W. (2010). The application of bioflocs technology to protect brine shrimp (*Artemia franciscana*) from pathogenic *Vibrio harveyi*. *Journal of applied microbiology*, 109(5), 1643-1649.
- Dauda, A. B., Romano, N., Ebrahimi, M., Teh, J. C., Ajadi, A., Chong, C. M., ... & Kamarudin, M. S. (2018). Influence of carbon/nitrogen ratios on biofloc production and biochemical composition and subsequent effects on the growth, physiological status and disease resistance of African catfish (*Clarias gariepinus*) cultured in glycerol-based biofloc systems. *Aquaculture*, 483, 120-130.
- De Schryver, P., Crab, R., Defoirdt, T., Boon, N., & Verstraete, W. (2008). The basics of bioflocs technology: the added value for aquaculture. *Aquaculture*, 277(3-4), 125-137.
- DoF .2003 .Brief on Department of Fisheries Bangladesh. Department of Fisheries, Ministry of Fisheries and Livestock, Dhaka, Bangladesh.
- DoF .2005 .Fishery Statistical Yearbook of Bangladesh 2003–2004. Fisheries Resources Survey System, Department of Fisheries, Ministry of Fisheries and Livestock, MatshyaBhaban, Dhaka, 46 pp.
- DPIF (1997). Marine Farming Development Plans for Tasmania D'Entrecasteaux Channel. Department of Primary Industry and Fisheries, Tasmania.
- EAO. Salmon Aquaculture Review. Environmental Assessment Office, British Columbia. <http://www.eao.gov.bc.ca/>
- Effendy, I., Al Deen, S., & Chithambaran, S. (2016). Semi Intensive and Semi Biofloc Methods for the Culture of Indian White Prawn, *Fenneropenaeus indicus* in High-density Polyethylene Liner Ponds. *HAYATI Journal of Biosciences*, 23(3), 106-110.
- E-Jahan, K. M., Ahmed, M., & Belton, B. (2017). The impacts of aquaculture development on food security: lessons from Bangladesh. *Aquaculture research*, 41(4), 481-495.
- Ekasari, J., Rivandi, D. R., Firdausi, A. P., Surawidjaja, E. H., Zairin Jr, M., Bossier, P., & De Schryver, P. (2015). Biofloc technology positively affects Nile tilapia (*Oreochromis niloticus*) larvae performance. *Aquaculture*, 441, 72-77.

- Emerenciano, M., Ballester, E. L., Cavalli, R. O., & Wasielesky, W. (2012). Biofloc technology application as a food source in a limited water exchange nursery system for pink shrimp *Farfantepenaeus brasiliensis* (Latreille, 1817). *Aquaculture research*, 43(3), 447-457.
- FAO, F. (2016). The State of World Fisheries and Aquaculture. Contributing to Food Security and Nutrition for All.
- Ferreira, G. S., Bolívar, N. C., Pereira, S. A., Guertler, C., do Nascimento Vieira, F., Mourião, J. L. P., & Seiffert, W. Q. (2015). Microbial biofloc as source of probiotic bacteria for the culture of *Litopenaeus vannamei*. *Aquaculture*, 448, 273-279.
- Gavin, J. J., & Cummings, D. P. (1989). Enumeration of microorganisms. *Practical Handbook of Microbiology*, 307.
- Ghose, B. (2014). Fisheries and aquaculture in Bangladesh: Challenges and opportunities. *Annals of Aquaculture and Research*, 1(1), 1-5.
- Grove, D. C., & Randall, W. A. (1955). Assay methods of antibiotics.
- Hajna, A. A., & Perry, C. A. (1943). Comparative Study of Presumptive and Confirmative Media for Bacteria of the Coliform Group and for Fecal Streptococci*. *American Journal of Public Health and the Nations Health*, 33(5), 550-556.
- Hargreaves J. A., 2013 Biofloc production systems for aquaculture. SRAC Publication number 4503, 12 pp.
- Hari, B., Kurup, B. M., Varghese, J. T., Schrama, J. W., & Verdegem, M. C. J. (2004). Effects of carbohydrate addition on production in extensive shrimp culture systems. *Aquaculture*, 241(1-4), 179-194.
- Heijtz, R. D., Wang, S., Anuar, F., Qian, Y., Björkholm, B., Samuelsson, A., ... & Pettersson, S. (2011). Normal gut microbiota modulates brain development and behavior. *Proceedings of the National Academy of Sciences*, 108(7), 3047-3052.
- Herigstad, B., Hamilton, M., & Heersink, J. (2001). How to optimize the drop plate method for enumerating bacteria. *Journal of Microbiological Methods*, 44(2), 121-129.

- Hussain, A. S., Mohammad, D. A., Ali, E. M., & Sallam, W. S. (2014). Nutrient Optimization for the Production of Microbial Flocs in Suspended Growth Bioreactors. *J Arabian Aquac Soc*, 9(1), 113-129.
- Hutabarat, J., Prayitno, S. B., & Darmanto, Y. S. (2013). The effect of different C: N and C: P ratio of media on the content of polyhydroxybutyrate in biofloc inoculated with bacterium *Bacillus cereus*. *Journal of Coastal Development*, 16(2), 114-120.
- ICLARM. (2002) .Profile of key aquaculture technologies and fishing practices, Bangladesh (Component 1). In: Status of the research component of the project "Strategies and options for increasing and sustaining fisheries and aquaculture production to benefit poor households in Asia". ADB-RETA 5945 Inception Report.
- Kim, S. K., Pang, Z., Seo, H. C., Cho, Y. R., Samocha, T., & Jang, I. K. (2014). Effect of bioflocs on growth and immune activity of Pacific white shrimp, *Litopenaeus vannamei* postlarvae. *Aquaculture Research*, 45(2), 362-371.
- Kumar, V. S., Pandey, P. K., Anand, T., Bhuvaneswari, G. R., Dhinakaran, A., & Kumar, S. (2018). Biofloc improves water, effluent quality and growth parameters of *Penaeus vannamei* in an intensive culture system. *Journal of environmental management*, 215, 206-215.
- Liu, L., Hu, Z., Dai, X., & Avnimelech, Y. (2014). Effects of addition of maize starch on the yield, water quality and formation of bioflocs in an integrated shrimp culture system. *Aquaculture*, 418, 79-86.
- Luis-Villaseñor, I. E., Voltolina, D., Audelo-Naranjo, J. M., Pacheco-Marges, M. R., Herrera-Espericueta, V. E., & Romero-Beltrán, E. (2015). Effects of biofloc promotion on water quality, growth, biomass yield and heterotrophic community in *Litopenaeus vannamei* (Boone, 1931) experimental intensive culture. *Italian Journal of Animal Science*, 14(3), 3726.
- Magondu, E. W., Charo-Karisa, H., & Verdegem, M. C. J. (2013). Effect of C/N ratio levels and stocking density of *Labeo victorianus* on pond environmental quality using maize flour as a carbon source. *Aquaculture*, 410, 157-163.

- Manan, H., Moh, J. H. Z., Kasan, N. A., Suratman, S., & Ikhwanuddin, M. (2017). Identification of biofloc microscopic composition as the natural bioremediation in zero water exchange of Pacific white shrimp, *Penaeus vannamei*, culture in closed hatchery system. *Applied Water Science*, 7(5), 2437-2446.
- Maslowski, K. M., & Mackay, C. R. (2010). Diet, gut microbiota and immune responses. *Nature immunology*, 12(1), 5.
- Megahed, M. E. (2010). The effect of microbial biofloc on water quality, survival and growth of the green tiger shrimp (*Penaeus semisulcatus*) fed with different crude protein levels. *Journal of the Arabian Aquaculture Society*, 5(2), 119-142.
- Meyer, F. P. (1991). Aquaculture disease and health management. *Journal of animal science*, 69(10), 4201-4208.
- Miao, S., Zhu, J., Zhao, C., Sun, L., Zhang, X., & Chen, G. (2017). Effects of C/N ratio control combined with probiotics on the immune response, disease resistance, intestinal microbiota and morphology of giant freshwater prawn (*Macrobrachium rosenbergii*). *Aquaculture*, 476, 125-133.
- Mook, W. T., Chakrabarti, M. H., Aroua, M. K., Khan, G. M. A., Ali, B. S., Islam, M. S., & Hassan, M. A. (2012). Removal of total ammonia nitrogen (TAN), nitrate and total organic carbon (TOC) from aquaculture wastewater using electrochemical technology: A review. *Desalination*, 285, 1-13.
- Naylor, R. L., Goldburg, R. J., Primavera, J. H., Kautsky, N., Beveridge, M. C., Clay, J., ... & Troell, M. (2000). Effect of aquaculture on world fish supplies. *Nature*, 405(6790), 1017.
- NPI National Pollutant Inventory. (2001) Emission estimation technique manual for aggregated emissions from temperate water finfish aquaculture. Environment Australia, June 2001.
- Pacheco-Vega, J. M., Cadena-Roa, M. A., Leyva-Flores, J. A., Zavala-Leal, O. I., Pérez-Bravo, E., & Ruiz-Velazco, J. M. (2018). Effect of isolated bacteria and microalgae on the biofloc characteristics in the Pacific white shrimp culture. *Aquaculture Reports*, 11, 24-30.

- Paturau, J.M. 1982. Alternative Uses of Sugarcane and Its Byproducts In Agroindustries.
- Pearson, T.H. & Rosenberg, R. (1979). Macrobenthic succession in relation to organic enrichment and the pollution of the marine environment. *Oceanography and Marine Biology Annual Review*. 16: 229-311
- Pérez-Fuentes, J. A., Pérez-Rostro, C. I., & Hernández-Vergara, M. P. (2013). Pond-reared Malaysian prawn *Macrobrachium rosenbergii* with the biofloc system. *Aquaculture*, 400, 105-110.
- Rajkumar, M., Pandey, P. K., Aravind, R., Vennila, A., Bharti, V., & Purushothaman, C. S. (2016). Effect of different biofloc system on water quality, biofloc composition and growth performance in *Litopenaeus vannamei* (Boone, 1931). *Aquaculture Research*, 47(11), 3432-3444.
- Ray, A. J., Seaborn, G., Vinatea, L., Browdy, C. L., & Leffler, J. W. (2012). Effects of Biofloc Reduction on Microbial Dynamics in Minimal-exchange, Superintensive Shrimp, *Litopenaeus vannamei*, Culture Systems. *Journal of the World Aquaculture Society*, 43(6), 790-801.
- Reno, P. W. (1998). Factors involved in the dissemination of disease in fish populations. *Journal of Aquatic Animal Health*, 10(2), 160-171.
- Ringø, E., Olsen, R. E., Gifstad, T. Ø., Dalmo, R. A., Amlund, H., HEMRE, G. I., & Bakke, A. M. (2010). Prebiotics in aquaculture: a review. *Aquaculture Nutrition*, 16(2), 117-136.
- Ritz, D. A., Lewis, M. E., & Shen, M. (1989). Response to organic enrichment of infaunal macrobenthic communities under salmonid seacages. *Marine Biology*, 103(2), 211-214.
- Rolando Y. Wee, (2017). Top 15 Countries For Aquaculture Production. Aquaculture Equipment Supplier - Turnkey Project for Fish Farm Bankable Project, Equipment Supply, and Installation Service for Fish Farms asaquaculture.com.
- Schveitzer, R., Arantes, R., Costódio, P. F. S., do Espírito Santo, C. M., Arana, L. V., Seiffert, W. Q., & Andreatta, E. R. (2013). Effect of different biofloc levels on microbial activity,

- water quality and performance of *Litopenaeus vannamei* in a tank system operated with no water exchange. *Aquacultural Engineering*, 56, 59-70.
- Shamsuzzaman, M. M., Islam, M. M., Tania, N. J., Al-Mamun, M. A., Barman, P. P., & Xu, X. (2017). Fisheries resources of Bangladesh: Present status and future direction. *Aquaculture and Fisheries*. Aquaculture and Fisheries, Volume 2, Issue 4, Pages 145-156.
- Silva, U. L., Falcon, D. R., da Cruz Pessôa, M. N., & de Souza Correia, E. (2017). CARBON SOURCES AND C:N RATIOS ON WATER QUALITY FOR NILE TILAPIA FARMING IN BIOFLOC SYSTEM. *Rev. Caatinga, Mossoró*, 30(4), 1017-1027.
- Subasinghe, R., Soto, D., & Jia, J. (2009). Global aquaculture and its role in sustainable development. *Reviews in Aquaculture*, 1(1), 2-9.
- Swanson, K. M. J., Petran, R. L., & Hanlin, J. H. (2001). Culture methods for enumeration of microorganisms. *Compendium of methods for the microbiological examination of foods*, 4th ed. American Public Health Association, Washington, DC, 53-62.
- Tapia-Paniagua, S., Lobo, C., Moreno-Ventas, X., de la Banda, I. G., Morínigo, M. A., & Balebona, M. C. (2014). Probiotic supplementation influences the diversity of the intestinal microbiota during early stages of farmed Senegalese sole (*Solea senegalensis*, Kaup 1858). *Marine biotechnology*, 16(6), 716-728.
- Taw N (2010) Biofloc technology expanding at white shrimp farms. *Global Advocate* may/june, 24-26
- Vicente Mabote, Delfina Muiocha, Manecas Francisco Baloi, (2017). Cultivation of black tiger shrimp *Penaeus monodon* (Fabricius 1798) at the nursery stage in biofloc technology (BFT) system. *African Journal of Fisheries Science*, Vol. 5(7), pp. 267-272.
- Wei, Y., Liao, S. A., & Wang, A. L. (2016). The effect of different carbon sources on the nutritional composition, microbial community and structure of bioflocs. *Aquaculture*, 465, 88-93.

- White, P. (2013). Environmental consequences of poor feed quality and feed management. *On-farm feeding and feed management in aquaculture, FAO Fisheries and Aquaculture Technical Paper*, (583).
- Woo, P. T. (1992). Immunological responses of fish to parasitic organisms. *Annual review of fish diseases*, 2, 339-366.
- Xu, W. J., Morris, T. C., & Samocha, T. M. (2016). Effects of C/N ratio on biofloc development, water quality, and performance of *Litopenaeus vannamei* juveniles in a biofloc-based, high-density, zero-exchange, outdoor tank system. *Aquaculture*, 453, 169-175.
- Yuniartik, M., Hariati, A. M., & Fadjar, M. (2015). Effect of Biofloc on Feed Efficiency and Growth of Pacific White Shrimp, *Litopenaeus vannamei* (Boone, 1932).
- Zeng, S., Huang, Z., Hou, D., Liu, J., Weng, S., & He, J. (2017). Composition, diversity and function of intestinal microbiota in pacific white shrimp (*Litopenaeus vannamei*) at different culture stages. *PeerJ*, 5, e3986.
- Zeng, Y., Ma, Y., Wei, C., Jiao, N., Tang, K., Wu, Z., & Jian, J. (2010). Bacterial diversity in various coastal mariculture ponds in Southeast China and in diseased eels as revealed by culture and culture-independent molecular techniques. *Aquaculture Research*, 41(9).
- Zhao, P., Huang, J., Wang, X. H., Song, X. L., Yang, C. H., Zhang, X. G., & Wang, G. C. (2012). The application of bioflocs technology in high-intensive, zero exchange farming systems of *Marsupenaeus japonicus*. *Aquaculture*, 354, 97-106.

Chapter Seven

APPENDICES

7 Appendices

7.1 Appendix-1

Table 7.1 Composition of SF Broth media for *E. faecalis* bacteria culture developed by Hajna and Perry (1943):

Ingredients	gm /Liter
Peptic Digest of Casein	20.0
Dextrose	5.0
Sodium Chloride	5.0
Dipotassium Phosphate	4.0
Monopotassium Phosphate	1.5
Sodium Azide	0.5
Bromcresol Purple	0.032
Total	36.032



Table 7.2 Composition of Clostridial Agar (M154) media for *Clostridium* spp. bacteria culture:

Ingredients	gm / Litre
Casein enzymichydrolysate	10.00
Beef extract	10.00
Yeast extract	3.00
Dextrose	5.00
Sodium chloride	5.00
Sodium acetate	3.00
Starch, soluble	1.00
L-Cysteine hydrochloride	0.50
Agar	13.50
Total	51.00

Table 7.3 Composition of Lactobacillus MRS Agar medium for *Lactobacillus* spp. bacteria culture:

Ingredients	gm / Litre
Proteose peptone	10.00
Beef extract	10.00
Yeast extract	5.00
Dextrose	20.00
Polysorbate 80	1.00
Ammonium citrate	2.00
Sodium acetate	5.00
Magnesium sulphate	0.10
Manganese sulphate	0.05
Dipotassium phosphate	2.00
Agar	12.00
Total	67.15

Table 7.4 Composition Broth Medium I (Tetrathionate Bile Brilliant Green Broth media) for *Salmonella* spp. bacteria culture:

Ingredients	Gms/Litre
Peptone	8.600
Ox-bile dried	8.000
Sodium chloride	6.400
Calcium carbonate	20.000
Potassium tetrathionate	20.000
Brilliant green	0.070
Total	63.07

7.2 Appendix- 2

PHOTO GALLERY



Figure 1. Floc Development in culture tank

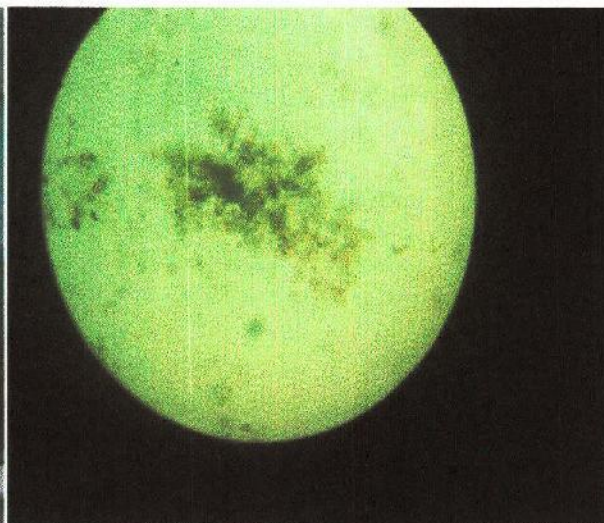


Figure 2. Microscopic view of Floc

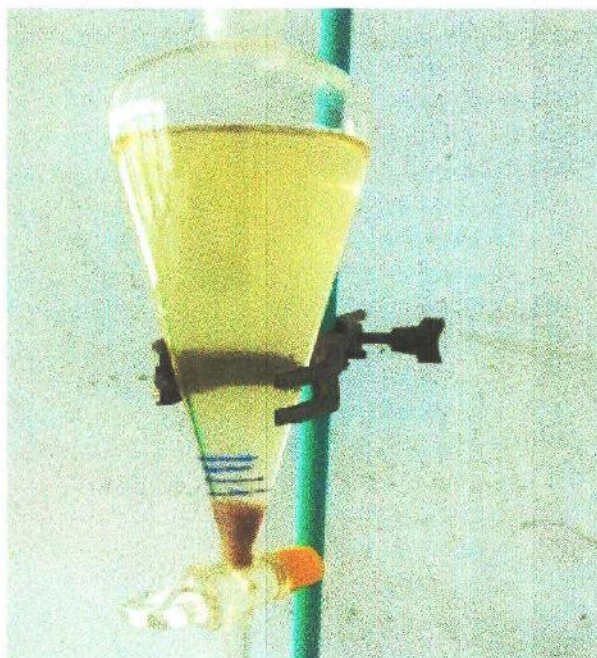


Figure 3. Floc Measurement in cone

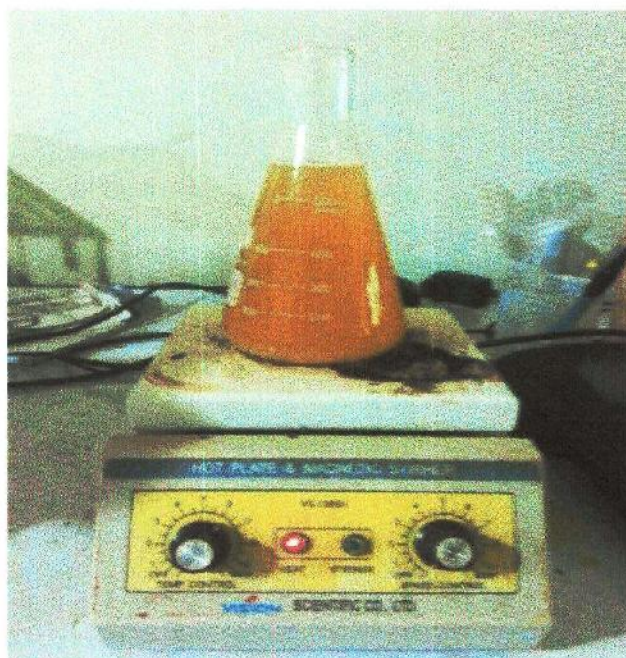


Figure 4. Media preparation for bacterial culture

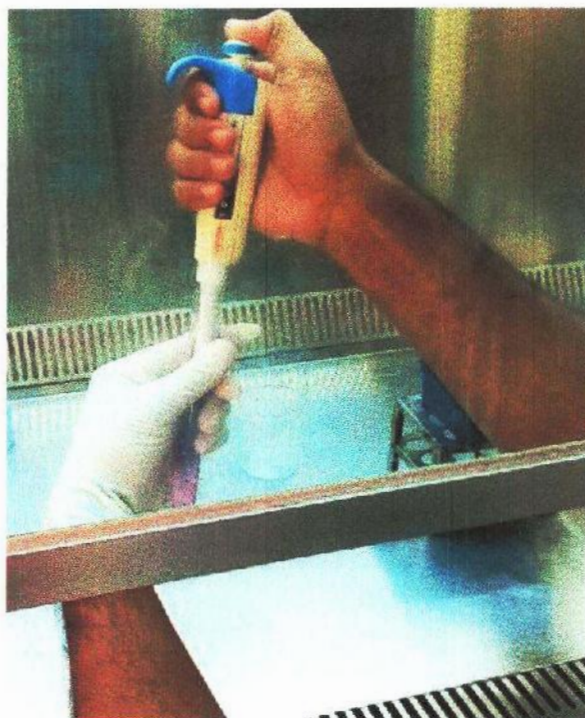


Figure 5. Collection of stock solution with micro pipette in bio-safety cabinet



Figure 6. Mixing of sample with culture media in Vortex mixer



Figure 7. Preparation of plate with Media



Figure 8. Sample pouring in plate

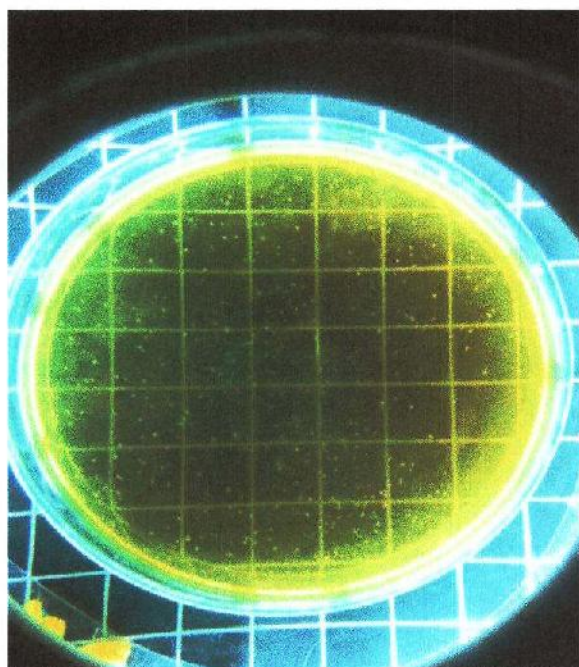


Figure 9. Plate culture of *Lactobacillus* spp.

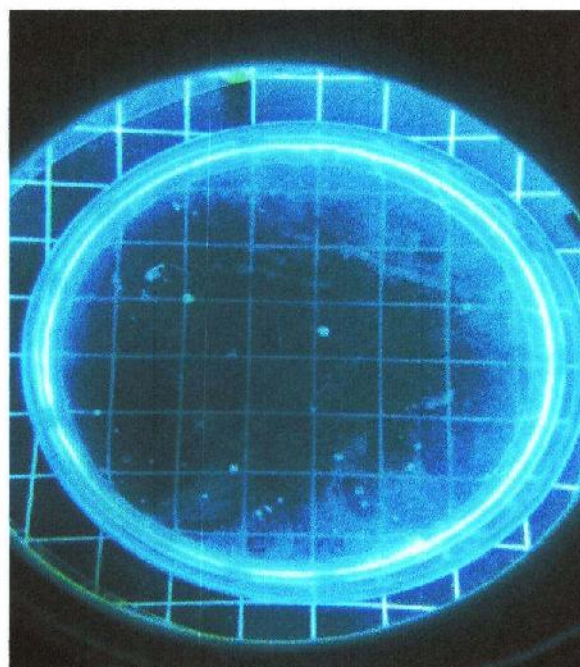


Figure 10. Plate culture of *Clostridium* spp.



Figure 11. Tube culture of *Enterococcus* spp.



Figure 12. Tube culture of *Salmonella* spp.

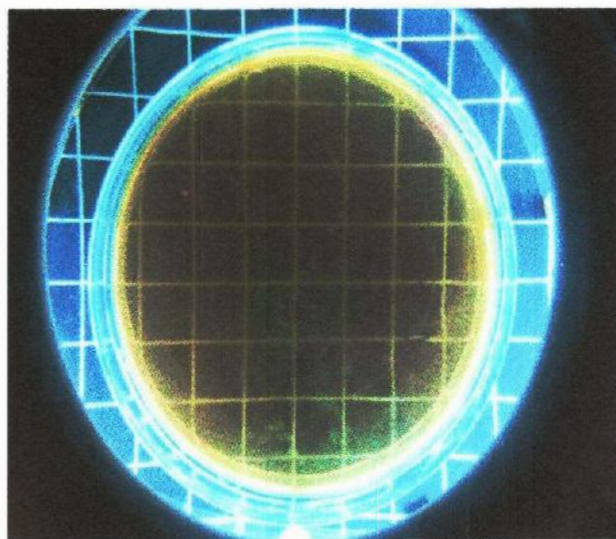


Figure 13. Plate culture of *Salmonella* spp.

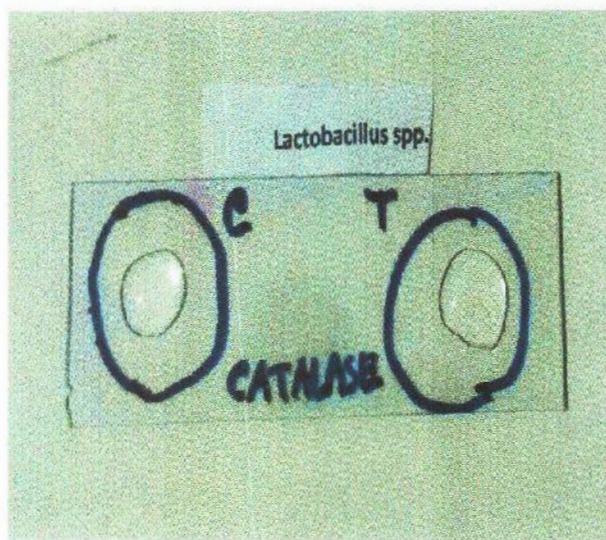


Figure 14. Catalase test



Figure 15. Indole test



Figure 16. SIM test

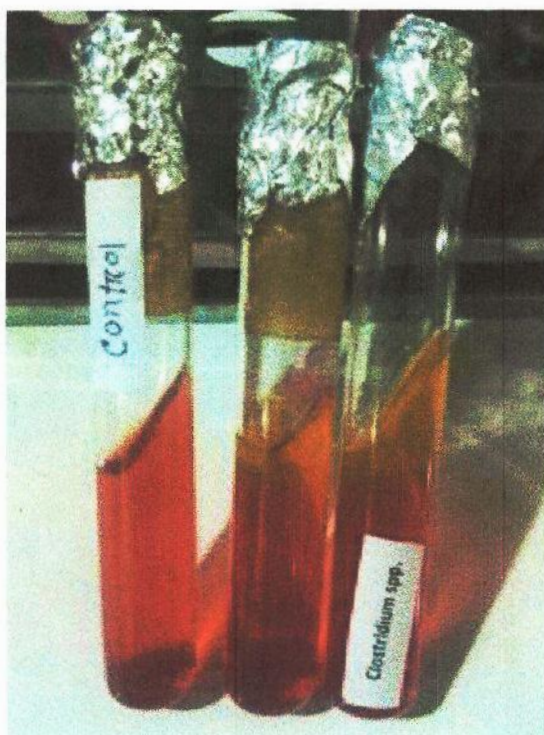


Figure 17. TSI test



Figure 18. Arabinose test

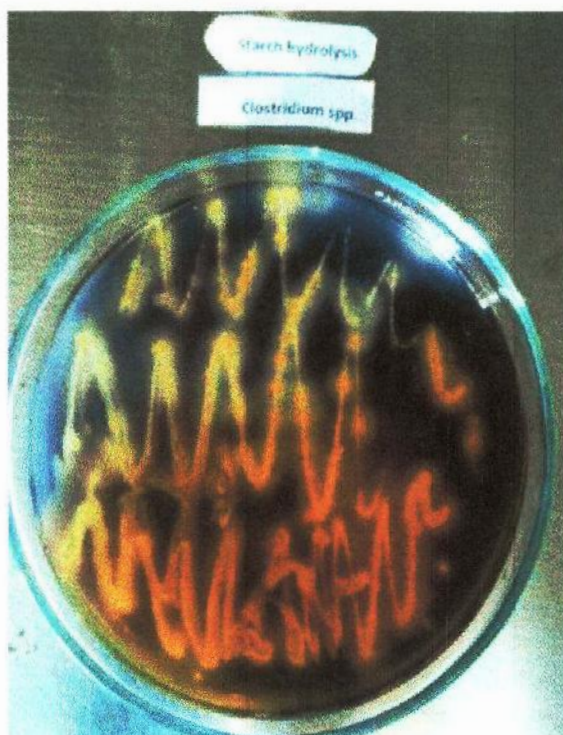


Figure 19. Starch Hydrolysis test



Figure 20. Sorbitol test